PHYTOCHEMICAL INVESTIGATION OF A FEW PLANTS

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FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN CHEMISTRY

By

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FEBRUARY 1998

To My Husband and Son

CERTIFICATE

This is to certify that this Thesis is an authentic record of the research work carried out by **Mrs. Bindu T.K.**, in the Department of Chemistry, University of Calicut, under my guidance and supervision in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry, under the Faculty of Science of the University of Calicut and that no part thereof has been presented earlier for any other degree.

It is also certified that Mrs. Bindu T.K. has passed the Ph.D. Preliminary Qualifying Examination in 1994.

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DECLARATION

I hereby declare that this Thesis is an authentic record of original research work carried out by me under the supervision of Dr. P. Mohamed Shafi and no part of this has previously formed the basis for the award of any degree or diploma as stipulated in the statutes of Calicut University.

Bindu T.K
16/2/09
BINDU T.K.

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Bindu T.K

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INTRODUCTION

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I N T R O D U C T I O N

Ever since the birth of man the plant kingdom has provided him with food, shelter and drugs. As our knowledge grew, especially in the twentieth century, the utility of plant ingredients could be studied in depth. Plants are sophisticated factories where a variety of chemical compounds are manufactured. They are now recognized as a potent renewable souce of raw materials for the production of a large variety of chemicals.

Synthetic chemicals in the form of drugs, pesticides etc. have been successful in combating diseases of man, his domestic animals and plant crops. However, their side effects are of major concern in the modern world. More over many of the insects and microorganisms have developed resistance to most of these chemicals. The nature of chemicals employed in the future has to be more selective and evnviornmentally safe.Hence we look to nature as an ally and resource in finding new strategies to combat diseases. In this respect plants serve as a vast reservoir of biodegradable chemicals, many of which may have evolved in the defence of predators.

Phytochemical studies have become more encouraging with

the advent of modern techniques and instrumentation. Hitherto impossible separations have become possible with the improved chromatographic techniques. The revolutionary advances in the field of spectroscopy and computers have made structural elucidation more simple and reliable.

In this work two plants, Hibiscus furcatus and Uvaria narum have been investigated for their chemical constitutents. Uvaria narum has also been studied for its antifungal activity against Phytophthora capsici, the foot rot pathogen of pepper.

The Thesis comprises of five chapters. Chapter I has two sections, the first being a review on Hibiscus species and the second deals with chemical investigation on Hibiscus furcatus. A review of the work done on Annonaceae family is presented in section 1 of the chapter II while section 2 of it deals with the investigation of Uvaria narum for its chemical constituents. Chapter III and IV deal with the studies on essential oils of the leaf and root bark respectively of Uvaria narum. The first section of chapter V describes, the antifungal property of Uvaria narum constituents on phytophthora capsici. The second section of this chapter deals with antifungal activity and structure-activity relationships.

In chapters I and II the isolation procedures of the compounds are given in 'materials and methods' section. Therefore to avoid repetition isolation procedures are not included in the experimental part. However, derivatisation procedures are included in the experimental section of chapter I.

In chapter III and IV the isolation of essential oils are given in detail in the 'materials and methods' section. Hence these chapters do not have an experimental section.

As chapter V involves a series of experiments they are presented seperately in the experimental section.

SECTION 1: THE PHYTOCHEMISTRY OF HIBISCUS SPECIES: A REVIEW

SECTION 2 : PHYTOCHEMICAL STUDIES CN HIBISCUS FURCATUS LEAVES

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CHAPTER I

SECTION 1: THE PHYTOCHEMISTRY OF HIBISCUS SPECIES-A REVIEW

Introduction

Hibiscus is one of the genera of the large family Malvaceae, which comprises about 100 genera and more than 2000 species. They are found in tropics and subtropics as herbs or shrubs, rarely trees most of them having showy flowers. About 160 species of this genus are identified. Some members of this genus are reported to be medicinally as well as economically important. Seeds of H. esculentus, H. manihot, H. sabdariffa etc. yield edible oils. The fatty oils obtained from H. cannabinus is used for the manufacture of soaps and varnishes. Seed oils of some species like H. abelmoschus also known as Musk seed oil or Ambrette seed oil are used in perfumery. Many members of this genus are used for the manufacture of fibres. H. esculentus gives a representative mucilage called Okra mucilage R which have better sustaining characteristics than even widely used synthetic materials like methyl cellulose.[1] Seeds of H. sabdariffa which is widely cultivated in India have remarkable coagulating property when applied to turbid water.[2]

Some species are reported to be medicinally important[3,4]. Infusion, decoction etc. derived from some of them are used for many therapeutic purposes. Various parts of the plant, H. mutabilis, commonly called Guliajaib, are used medicinally. Leaves for example is used for cough, menorrhagia and wounds caused by burns and scalds. A mucilaginous preparation from the fruits of Hibiscus esculentus has found application as a plasma replacement or blood volume expander. Another member, H. rosa-sinensis, commonly known as China rose plant, appears to have potent contraceptive effect. In Ayurvedic literature, the flowers of this plant are attributed to possess antifertility effect[5]. Ether soluble portion of the water insoluble fraction of the benzene extract showed the most significant antiimplantation and abortifacient activity[6-9]. Flowers are not found to have any such side effects as would warrant its withdrawal. This is a welcome feature considering the well-known side effects of currently available contraceptives which are mostly hormonal in nature. A number of compounds like cyanidin, quercetin, hentriacontane, cyanidin diglucoside, calcium oxalate, tannins, thiamine, riboflavin, niacin and ascorbic acid have been reported from this plant. But none has found to have antifertility activity. The leaves, flowers, fruits and

seeds of various Hibiscus species are of definite food value.

H. sabdariffa calyx is reported to have antispasmodic potential.[10] This plant is also studied for its estrogen like activity.[11] Rats fed on H. sabdariffa oil showed inferior growth and reproductive performances. H. sabdariffa also shows antimicrobial activity against many bacteria.[12] The seed of Hibiscus abelmoschus is also called Viper seed or Snake seed, since it is used to cure snake bite. The seed contained no alkaloid and no glycoside, but had about 43% adsorbent capacity of activated charcoal.[13] The action of Viper seed may be that of finely dispersed emulsions which inactivated cobra venom by adsorption. Chemical analysis of the seed gave the following results by weight. Moisture 11.4%, ash 5.3%, starch 13.35%, protein 2.3%, crude fibre 31.46% and oil 14.5%. Leaves of H. esculentus was shown to have antibiotic action[14] against Escherichia coli and the bark of H. syriacus showed antifungal characteristics[15].

Malvaceae family as a whole and Hibiscus genus in particular, are rich sources of secondary metabolites of varying categories. Most of the phytochemical works in various Hibiscus species are concentrated on flowers. Studies on leaves, root bark etc. are less. The important

classes of compounds isolated from them are described in the following paragraphs.

Flavonoids

Flavonoids are a group of natural plant pigments. Structurally they are pyrone pigments. Numerous flavonoids have been isolated from various plants belonging to Hibiscus genus of the Malvaceae family. Most of the flavonoids isolated from Hibiscus species are glycosides of quercetin, kaempferol and gossypetin. Some flavonoids like gossypin, quercetin-3-robinobioside, sabdaretin and hyperin are of pharmacological interest. The decoction of the flower extract of H. sabdariffa containing several flavonoids like sabdaretin, gossypin etc. acts as a hypotensive agent.[16] Quercetin-3-robinobioside, quercetin-3-glucoside, hyperin, quercetin and myricetin were isolated from Abelmoschus manihot (H. manihot), flowers of which are used in the treatment of chronic bronchitis and tooth ache[17]. A new flavanone glycoside, Naringenin-5,7-dimethyl ether-4'-O-β-D-xylopyranosyl-β-D-arabinopyranoside in addition to various flavonoids like isoquercitrin and quercetin-3-sambubioside have been isolated from H. mutabilis, which is used medicinally[18]. Flavonoids from various Hibiscus species are given in Table I.1.

Anthocyanins

Anthocyanins are another group of plant pigments based on benzopyrilium or flavylium chloride structure. These pigments are responsible for the intense colour of flowers, leaves, and fruits of various plants. Numerous shades of colours are due to same skeletal structure, but different substitution. A number of anthocyanins have been isolated from the flowers and leaves of various Hibiscus species. Most of them are glycosides of cyanidin and delphinidin. Three new anthocyanidin malonylglycosides have been reported from H. syriacus[19], along with the known compounds like 3-O-glucosides of delphinidin, cyanidin, petunidin, malvidin and three known anthocyanidin 3-O-malonylglucosides.[20] Most common anthocyanin is cyanidin 3-sambubioside. Cyanidin 3-glucoside is found in large woody species and cyanidin 3-sophoroside is present mostly in plants like H. rosa-sinensis i.e. in the section Lilibiscus of Malvaceae family. Anthocyanins and anthocyanidins reported from various Hibiscus species are given in Table I.2.

Terpenoids and steroids

β-Sitosterol, the commonly occuring plant sterol has been isolated from various members of this genus. The seed

oil of H. sabdariffa is found to contain ergosterol, the most important mycosterol. The other sterols isolated from it are cholesterol, campesterol, stigmasterol, β-sitosterol and α-spinasterol[21]. Several monoterpenes like limonene, citral and phellandrene are isolated from the seed oil of H. cannabinus[22]. Triterpenoids like taraxeryl acetate are isolated from some members like H. rosa-sinensis. Several sesquiterpenoid quinones and related compounds (hibiscones A-D and hibiscoquinones A-D) are isolated from some other members[23,24]. Analysis of volatile oils of H. esculentus indicated the presence of about 148 compounds of which 16 were terpenes[25]. The terpenoids and steroids isolated from Hibiscus species are tabulated in Table I.3.

Sugars

Sugars may be present either in the bonded state ie, with hydroxyl groups present in the aglycones of flavonoids and anthocyanins or in the free state. The most common sugars are glucose, galactose, xylose, rhamnose and sucrose. Although glycosides like robinobioside, sophoroside and sambubioside have been reported from different Hibiscus species, free sugars robinobiose, sambubiose and sophorose haven't been reported from Hibiscus species. Sugars isolated from different Hibiscus species are listed in Table I.4.

Amino acids

Various amino acids are isolated from different species of Hibiscus genus of Malvaceae family. Recently, thiamine, riboflavin and niacin are isolated from H. rosa-sinensis. Amino acids isolated from this genus are given in Table I.5.

Fatty acids and related compounds

Fatty acids occur as glycerides in oils and fats and combined with flavonoids, anthocyanins and leucoanthocyanins as their esters. There is a co-occurence of cyclopropene acids and epoxy acids in the seed oils of Malvaceae (especially in the genus Hibiscus). Studies on the fatty acid constituents of Hibiscus have been mainly confined to seed oils in which palmitic, stearic, oleic and linoleic acids are the main compounds[26-28]. Citric acid, malic acid and tartaric acid occur in free state in plants like H. sabdariffa. The infusion of H. sabdariffa is a refreshing beverage. It has diuretic and chloretic effect, decreases the viscosity of blood and reduces blood pressure. It also reduces the intestinal peristalsis[29]. The stem bark of H. rosa-sinensis yielded lettuce seed germination inhibitors. The active constituents were four fatty acid methyl esters, 9-decynoate, 8-nonynoate, 10-oxo-11-octadecynoate and 8-oxo-

9-octadecynoate. The other two compounds isolated were, methyl-10-methylene-9-oxo-octadecanoate and methyl-9-methylene-8-oxo-heptadecanoate[30,31]. They are one of a few classes of aliphatic compounds possessing a terminal acetylenic linkage from higher plants. Two novel cycloprene acids sterulic and malvalic acids are isolated from the seeds of H. esculentus[32]. Two acetylenic compounds stearolic (9-octadecynoic) and 8-heptadecynoic acids are isolated from the seeds of H. syriacus. Two novel aliphatic enone ethers, methyl (E)-11-methoxy-9-oxo-nonadecenoate and (E)-10-methoxy-8-oxo-9-octadecenoate were also isolated from the stem bark of H. rosa-sinensis[33]. Various fatty acids and their derivatives isolated from Hibiscus genus are given in Table I.6.

Alcohols and Hydrocarbons

A large number of alcohols and hydrocarbons have been isolated from various members of this genus. They are listed in Table I.7 and Table I.8 respectively.

Miscellaneous compounds

In addition to the compounds mentioned above, several other classes of compounds have been isolated from many members of this genus. They include amines[34,35],

carbohydrates[36], carotenes[37], catechols[38,39], pentosans[40], phospholipids[41,42,43], polyphenols[44], polyuronides[45], ascorbic acid, proteins[36], proanthocyanidins[46,47] etc. The mucilage obtained from certain members like H. syriacus,[48] H. esculentus[49,50] are also studied.

Table I.1. Flavonoids of Hibiscus species

	Name	Reference	Source
1.	Cannabiscitrin	51	H. cannabinus
2.	Gossypetin	52,53,54	H. sabdariffa H. tiliaceus H. suratensis
3.	Gossypin	55,56,57, 58	H. vitifolius H. esculentus H. sabdariffa H. furcatus
4.	Gossypitrin	52,54,58, 59	H. sabdariffa H. suratensis H. furcatus
5.	Gossytrin	60	H. sabdariffa
6.	Gossypetin-8-(rhamnosyl glucoside)	56	H. esculentus
7.	Hibifolin	61	H. vitifolius
8.	Hibridin	62	Hybrid Hibiscus
9.	Hibiscatin	58	H. furcatus
10.	Hibiscetin	52	H. sabdariffa H. esculentus H. vitifolius
11.	Isoquircitrin	63,64	H. cannabinus H. mutabilis
12.	Kaempferol	53	H. tiliaceus
13.	Kaempferol-3-glucoside	63	H. cannabinus

	Name	Reference	Source
14.	Kaempferol-3-rhamnoside	63	H. cannabinus
15.	Kaempferol-3-galactoside	65	H. tiliaceus
16.	Kampferol-3-rhamnosyl glucoside	63	H. rosa-sinensis
17.	Kaempferol-3-β-rhamnopyranosido 7-α-rhamno-furanoside	66 -	H. cannabinus
18.	Kaempferol-7-α-L-rhamno-pyranoside	67	H. canabinus
19.	Kaempferol-3-xylosyl glucoside	57	H. rosa-sinensis
20.	Quercetin	17,52,53 67,68,69	Various Hibiscus species
21.	Quercetin-3-β-D-gluco-pyranoside	70	Hybrid Hibiscus
22.	Quercetin-3-D-galactoside	65	H. tiliaceus
23.	Quercetin-3-D-glucosyl glucoside	56	H. esculentus
24.	Quercetin-3-robinobioside	17	H. manihot
25.	Quercetin-3-Sambubioside	71	H. mutabilis

	Name	Reference	Source
26.	Quercetin-3,4'-(diglucosyl glucoside)	56	H. esculentus
27.	Quercetin-3,7-diglucoside	57	H. rosa-sinensis
28.	Quercetin-3'-glucoside	17	H. manihot
29.	Quercetin-4'-glucoside	56,64	H. esculentus H. mutabilis
30.	Quercetin-5-glucoside	56	H. esculentus
31.	Quercetin-5-rhamnosyl glucoside	56	H. esculentus
32.	Quercimeritrin	56,72	H. esculentus H. mutabilis
33.	Myricetin	17,64	H. sabdariffa H. manihot
34.	Myricetin-3'-glucoside	73	H. abelmoschus
35.	Rutin	63,66	H. cannabinus H. mutabilis
36.	Sabdaretin	52	H. sabdariffa
37.	Saponarin	74	H. syriacus
38.	Naringenin, 5,7-dimethyl ether-4'o-β-D-xylopyranosyl-β-D-arabino-pyranoside	18	H. mutabilis

Table I.2. Anthocyanins and anthocyanidins of Hibiscus species

	Name	Reference	Source
1.	Cyanin	57,64,71, 75	H. rosa-sinensis H. mutabilis
2.	Cyanidin	54,69,76, 77	H. rosa-sinensis H. mutabilis
3.	Chrysanthamin	68,78,79, 80,81,82, 83	Various Hibiscus species
4.	Cannabinin	81,84,85, 43	H. cannabinus
5.	Cyanidin-3-sambubioside	82,86	H. sabdariffa
6.	Cyanidin-3-sophoroside	83,86,87	Various Hibiscus species
7.	Cyanidin-3-(2-glucosyl-glucoside)	57	H. sabdariffa
8.	Cyanidin-3-sophoroside-5-glucoside	57	H. rosa-sinensis
9.	Cyanidin-3,4'-diglucoside	56	H. esculentus
10.	Cyanidin-3-rutinoside-5-glucoside	64	H. mutabilis
11.	Cyanidin-4'-glucoside	56	H. esculentus
12.	Delphinidin	78	H. sabdariffa
13.	Delphinidin-3-glucoside	78	H. sabdariffa

	Name	Reference	Source
14.	Delphinidin-3-sambubioside	82,86	H. sabdariffa
15.	Gossypicyanin	79,81,88	Hybrid Hibiscus
16.	Hirsutine	89,90	Hybrid Hibiscus
17.	Mytrillin	85,43	H. cannabinus
18.	Cyanidin-3-glucoside	91	H. sabdariffa

Table I.3. Steroids and Terpenoids of Hibiscus species

	Name	Reference	Source
1.	Campesterol	92	H. sabdariffa
2.	Cholesterol	92	"
3.	Ergosterol	92	"
4.	β-Sitosterol	21,63,65, 93,94,95, 96	Various Hibiscus species
5.	β-Sitosterol-β-D-glucoside	95	H. abelmoschus
6.	β-Sitosterol-β-D-galacto-pyranoside	97	H. sabariffa
7.	Stigmasterol	92	"
8.	α-Spinasterol	92	"
9.	Limonene	22	H. cannabinus
10.	Citral	22	"
11.	Phellandrene	22	"
12.	α-Terpenyl acetate	22,96	"
13.	Taraxeryl acetate	94	H. rosa-sinensis H. esculentus

Table I.4. Sugars of Hibiscus species

	Name	Reference	Source
1.	D-Glucose	22,96,98, 99,100,101 102,103	Various Hibiscus species
2.	Xylose	98,99,100 101,102, 104	H. abelmoschus H. ficulneus
3.	L-Arabinose	"	Various Hibiscus species
4.	L-Rhamnose	79,98,99 100,104	"
5.	Stachyose	105	H. cannabinus
6.	Raffinose	105	H. cannabinus
7.	Sucrose	22,96,105	H. esculentus H. cannabinus
8.	D-Galactose	96,98,99, 100	H. esculentus H. ficulneus
9.	Mannose	22,96	H. esculentus H. cannabinus
10.	Lactose	22,96	H. esculentus H. cannabinus
11.	Fructose	22,96	H. cannabinus

Table I.5. Amino acids of Hibiscus species

	Name	Reference	Source
1.	Alanine	97	H. cannabinus
2.	α-Amino adipic acid	106	Various Hibiscus species
3.	Arginine	97,107	H. esculentus H. cannabinus
4.	Aspartic acid	97,108	H. sabdariffa H. cannabinus
5.	Cystine	97	H. sabdariffa H. cannabinus
6.	Glutamic acid	97	H. sabdariffa
7.	Glycine	97	H. cannabinus
8.	Histidine	97	H. cannabinus H. esculentus
9.	Homoserine	106	Various Hibiscus species
10.	Isoleucine	97	"
11.	Leucine	97	"
12.	Lysine	97	"
13.	Methionine	97	"
14.	Phenylalanine	107	H. esculentus
15.	Proline	97	H. cannabinus
16.	Threonine	107	H. esculentus
17.	Tryptophan	97,107	H. esculentus H. cannabinus
18.	Tyrosine	97	H. cannabinus
19.	Valine	107	H. esculentus

Table I.6. Organic acids of Hibiscus species

	Name	Reference	Source
1.	Arachidic acid	93,96	H. esculentus
2.	Ascorbic acid	109	H. sabdariffa H. esculentus
3.	Citric acid	110,111	H. sabdariffa H. rosa-sinensis
4.	Cerotic acid	92	H. sabdariffa
5.	P-Coumaric acid	65,112	H. rosa-sinensis H. tiliaceus
6.	Fumaric acid	65	H. rosa-sinensis
7.	Hibiscus acid	113,114	H. sabdariffa
8.	Hydroxycitric acid	115,116	H. furcatus H. cannabinus
9.	Hexadecanoic acid	117	H. esculentus
10.	Linoleic acid	63,117	H. esculentus H. cannabinus H. sabdariffa
11.	Lignoceric acid	92	H. cannabinus
12.	Malic acid	110	H. sabdariffa
13.	Malvalic acid	118,119	H. rosa-sinensis
14.	Oleic acid	117	H. esculentus
15.	12,13-Epoxy oleic acid	117	H. esculentus

	Name	Reference	Source
16.	Oxalic acid	120	H. rosa-sinensis H. sabdariffa
17.	Palmitic acid	96,93, 115,117	Various Hibiscus species
18.	Stearic acid	"	"
19.	Sterulic acid	112,118, 119	H. rosa-sinensis H. caesius
20.	Tartaric acid	110,111	H. sabdariffa H. rosa-sinensis
21.	Stearolic acid (9-octa-decynoic acid)	28	H. syriacus
22.	8-Heptadecynoic acid	28	"
23.	8-Nonynoic acid	30,31	H. rosa-sinensis
24.	9-Decynoic acid	30,31	"
25.	Myristic acid	121	H. sabdariffa
26.	Palmitoleic acid	43	"
27.	cis-12,13-Epoxy-cis-9-octadecenoic acid	88	"

Table I.7. Alcohols of Hibiscus species

	Name	Reference	Source
1.	Methyl alcohol	96	H. esculentus
2.	Ethyl alcohol	22,96	H. esculentus H. cannabinus
3.	Isopropyl alcohol	96	H. esculentus
4.	3-Methyl-1-butanol	96	"
5.	Cyclohexanol	22,96	"
6.	Hexacosanol	22,111	H. cannabinus Hybrid Hibiscus
7.	Octacosanol	111	"
8.	Hexadecanol	111	"
9.	Octadecanol	111	"
10.	Triacontanol	92	H. cannabinus
11.	Dotriacontanol	92	H. cannabinus

Table I.8. Hydrocarbons of Hibiscus species

	Name	Reference	Source
1.	Hexacosane	92	H. cannabinus
2.	Heptacosane	92	"
3.	Octacosane	92	"
4.	Nonacosane	92	"
5.	Hentriacontane	92,111	H. cannabinus H. rosa-sinensis
6.	Dotriacontane	92,111	Hybrid Hibiscus
7.	Tritriacontane	92,111	"
8.	Tetratriacontane	92,111	"
9.	Pentatriacontane	92,111	"
10.	Hexatriacontane	92,111	"
11.	Heptatriacontane	92,111	"
12.	Octatriacontane	92,111	"
13.	Tetracontane	92	H. cannabinus

SECTION 2: PHYTOCHEMICAL STUDIES ON HIBISCUS FURCATUS LEAVES

Introduction

Hibiscus furcatus belongs to the section Furcaria of the family Malvaceae. This section is characterized by the prickly habit and epicalyx segments with an appendage on the inner surface. It is a trailing or suberect prickly herb, 2-5 feet high found in almost all parts of India, Bangladesh, Myanmer, Sri Lanka and South Africa. Its flowers are large, yellow with purple centre. This plant flowers during September-November and fruiting takes place during November-January. It is reported to be highly resistant to drought, remaining green even under prolonged dry periods.[122]

The leaves of this plant are acidic and eaten after cooking. They are said to improve digestion when eaten and are considered to be anthelmintic. The juice of leaves mixed with honey is applied in eye diseases. An infusion of the roots in water is used as a cooling drink in hot weather. A decoction of the root bark is given as a remedy for poisons and swellings and for cleansing kidneys.[123]

I.1 Work so far reported

Leaves of H. furcatus are acidic and

l-allohydroxycitric acid has been isolated from them.[115] A flavonol glycoside hibiscatin is reported[124] to be present in the flowers along with gossypin and gossypitrin. β-Sitosterol and friedelin are also isolated and identified from the leaves of this plant.[125]

I.2 MATERIALS AND METHODS

Plant material

The plant material used in this investigation was collected from Trissur district of Kerala, India and was authenticated by Dr. A.K. Pradeep, Department of Botany Calicut University, Kerala, where a voucher specimen has been deposited.

Melting point determination

All the melting points of the crystalline isolates were determined using Toshniwal Capillary Melting point apparatus, and are uncorrected.

Infra red absorption spectroscopy (IR)

The IR spectra of the isolates were recorded using Shimadzu FTIR-8101 A spectrometer and the spectra were determined as KBr pellets.

Nuclear Magnetic Resonance Spectroscopy (NMR)

The ^{1}H NMR spectra of the isolates were recorded at 360, 400 or 500 MHz in $CDCl_3$ using tetramethylsilane (TMS) as internal standard using Bruker spectrometers. ^{13}C NMR spectra were recorded at 63 MHz or 125 MHz in $CDCl_3$ using TMS as internal standard. The chemical shifts are reported in ppm (δ).

Electron impact mass spectra (EI MS)

The electron impact mass spectra (EI MS) were recorded on Jeol-D-300 mass spectrometer at ionisation energy 70 eV. High resolution mass spectrum was recorded using Finnigan MAT 95 under the same conditions as above.

Gas Chromatography-Mass Spectroscopy (GC-MS) Analysis

GC-MS Analysis of the isolated components (H_1 and H_5) were done using Varian 3400 GC with 0.3 μm fused silica column (Perkin Elmer Permaphase PVMS/54, I.D. 0.25 mm of about 22 m length).

Column Chromatography (CC)

Column chromatographic separation of the crude and semipurified extracts were carried out using silicagel

(Acme, 100-200 mesh) and neutral alumina (Qualigens). The columns were prepared as a slurry with suitable solvents and eluted with selected chromatographic solvents.

Preparative Thin Layer Chromatography (Pre-TLC)

Preparative thin layer chromatography of the semi purified components were carried out in silicagel-G (Acme) plates prepared using Stahl apparatus (layer thickness 1 mm).

Thin Layer Chromatographic Analysis (TLC)

Thin layer chromatographic plates were prepared using TLC grade silicagel-G (Acme), layer thickness 0.2 mm (prepared using Stahl apparatus).

Reagents

Liebermann-Burchard Reagent (For triterpenes and sterols)

The reagent was prepared as follows:

Spray solution

Acetic anhydride	-	5 mL
Conc. Sulphuric acid	-	5 mL
Ethanol	-	50 mL

Acetic anhydride and 97% sulphuric acid were mixed carefully and with cooling freshly before use and ethanol was added to that mixture with cooling.

Treatment of the plates : Heated to 110°C until maximal visualisation of the spots.

I.3 Extraction, fractionation and isolation of compounds from the leaves of Hibiscus furcatus

Dried and finely powdered leaves of H. furcatus (2 kg) were extracted repeatedly with petrolum ether (60-80°C, 3 x 6L). The combined extract was then concentrated under reduced pressure to about 500 mL. This extract was then adsorbed on 750 g of neutral alumina and packed in a column (3cm X 100 cm; d X l). The column was then eluted with benzene (2 L), 3:1 benzene-ethyl acetate (2 L) and then with ethyl acetate alone (1 L). The concentrated benzene and 3:1 benzene-ethyl acetate fractions were combined and subjected further to column chromatography on silicagel and eluted with solvents of increasing polarity viz. petroleum ether, benzene, different combinations of benzene-ethylacetate and ethylacetate in that order. Several 25 mL fractions were collected and each fraction checked by TLC. Identical portions were pooled together and the solvent boiled off. Different compounds isolated from the petroleum ether extract are given in Table 1.9.

Table 1.9. **Compounds isolated from the Petroleum ether extract of Hibiscus furcatus leaves**

Compound	Eluent composition	m.p.	Molecular mass
H_1	Petroleum ether	64°C	478
H_2	Benzene	262°C	426
H_3	3:1 Benzene-ethyl acetate	272°C	426
H_4	3:1 Benzene-ethyl acetate	139°C	414
H_5	3:1 Benzene-ethyl acetate	82°C	476

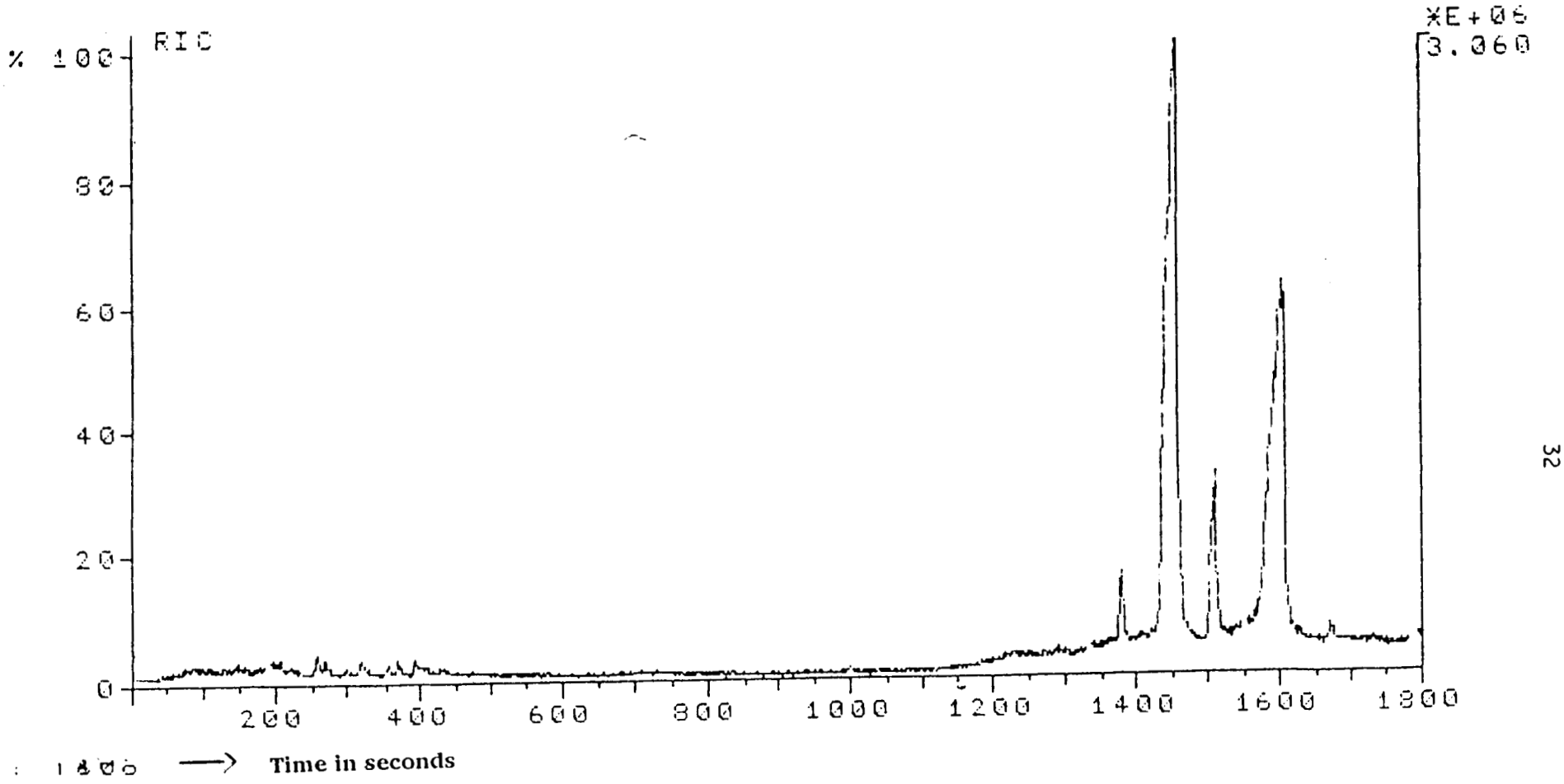
The residue after petroleum ether extraction was subjected to alcohol extraction. No crystalline compound could be isolated from it.

1.4. RESULTS AND DISCUSSION

1.4.1 Identification of H_1

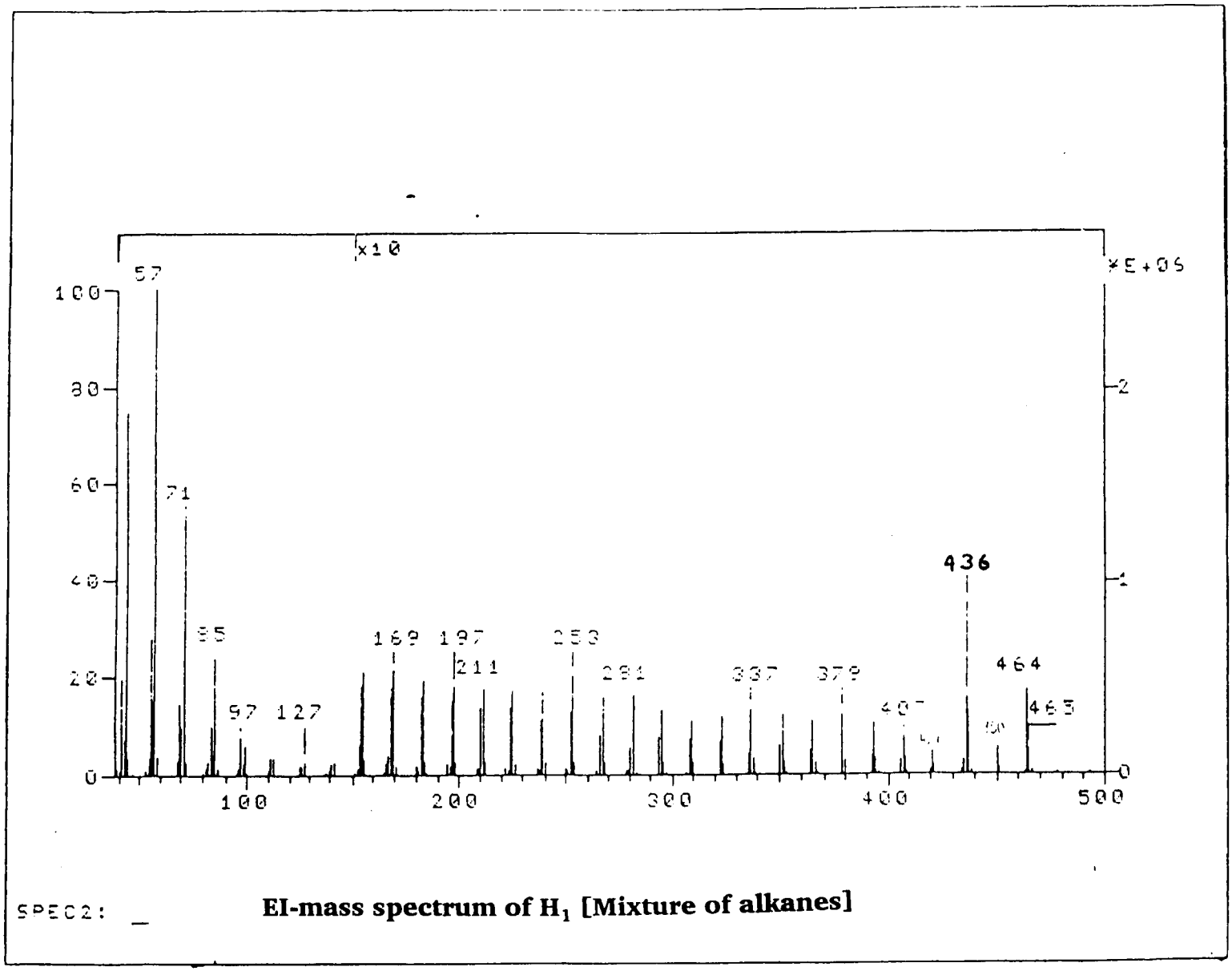
Component H_1 was obtained on elution with petroleum ether (60-80°C) as under I.3 and recrystallised from acetone as a white powdery substance (150 mg). It melted at 64°C. The IR spectrum gave characteristic absorptions indicating the presence of only C-H and C-C bonds, (2918.7, 2849.2, 1478.8, 1464.1 cm^{-1}). The absorption bands at 734 and 719

cm^{-1} were indicative of a long hydrocarbon chain. ^{1}H NMR Spectrum (absorptions in the region δ 0.9-1.65) and ^{13}C NMR spectrum (absorptions at δ 14.1, 22.69, 25.05, 25.45 and a number of absorption around 29 and another at 31.93) also supported the straight chain hydrocarbon nature. The EI mass spectrum of the component had a fragmentation pattern with a regular difference of 14 mass units and the base peak at, m/z 57 and the maximum m/z value at 478. The high intensity of peaks at m/z 464 and 436 did not conform to those of fragment ions but were indicative of a mixture of compounds. This doubt could be resolved by conducting GC-MS analysis of H_1. The temperature was programmed from $60^{o}C$ to $320^{o}C$ at a rate of $15^{o}C$/minute and then holding at $320^{o}C$ for 15 minutes. The carrier gas used was helium. Online acquisition and computation of mass spectral data were performed. The compounds were identified as straight chain C_{30}-C_{34} hydrocarbons, by comparison of their mass spectra with reference spectra in the mass spectral data base of the instrument. (The mass spectra of hentriacontane and tritria contane are provided). The major compounds present in the mixture were found to be hentriacontane, $C_{31}H_{64}$ (about 60% of the mixture), m/z at 436 and tritriacontane $C_{33}H_{68}$ (30%), m/z at 464. The compounds in minor quantities were triacontane ($C_{30}H_{62}$), dotriacontane ($C_{32}H_{66}$) and tetratriacontane ($C_{34}H_{70}$). The percentage composition was determined by the peak area measurement of GC.

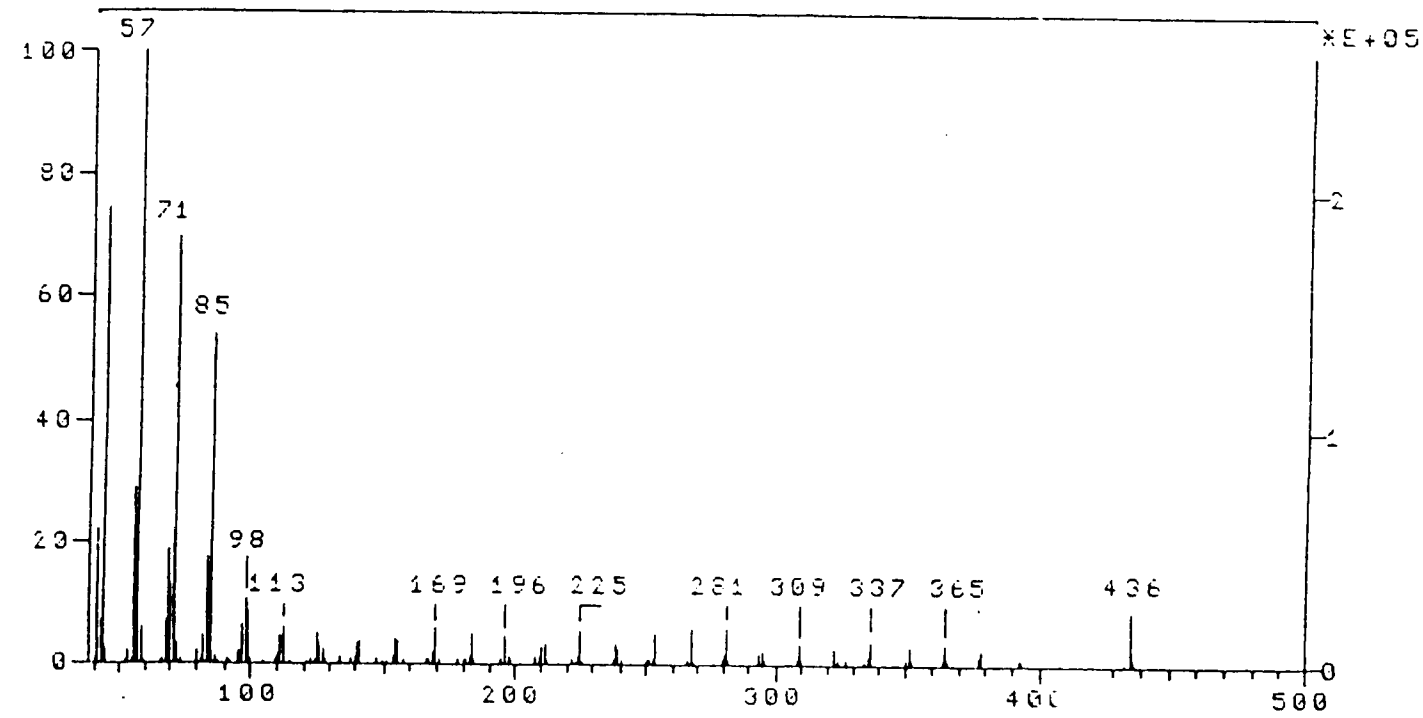


CHR02: _

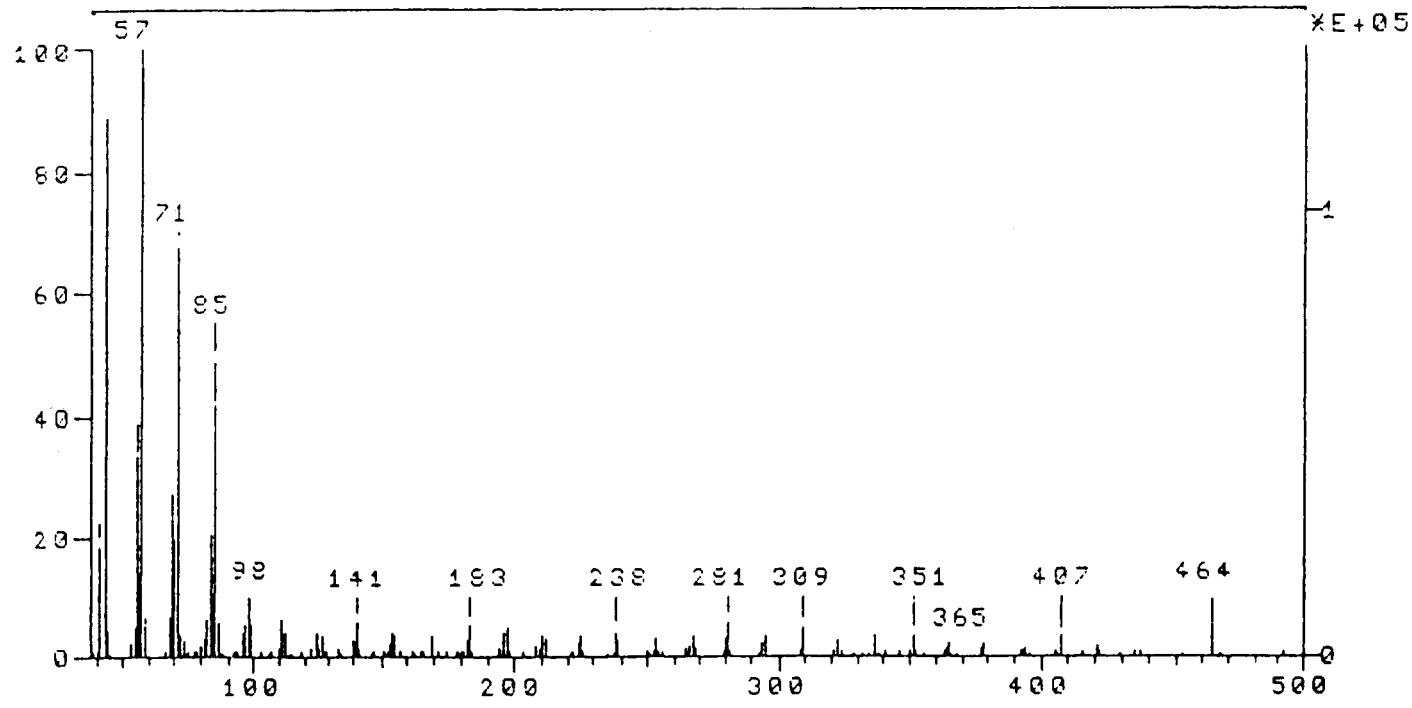
Gas Chromatogram of H_1 [Mixture of alkanes]



EI-mass spectrum of H_1 [Mixture of alkanes]

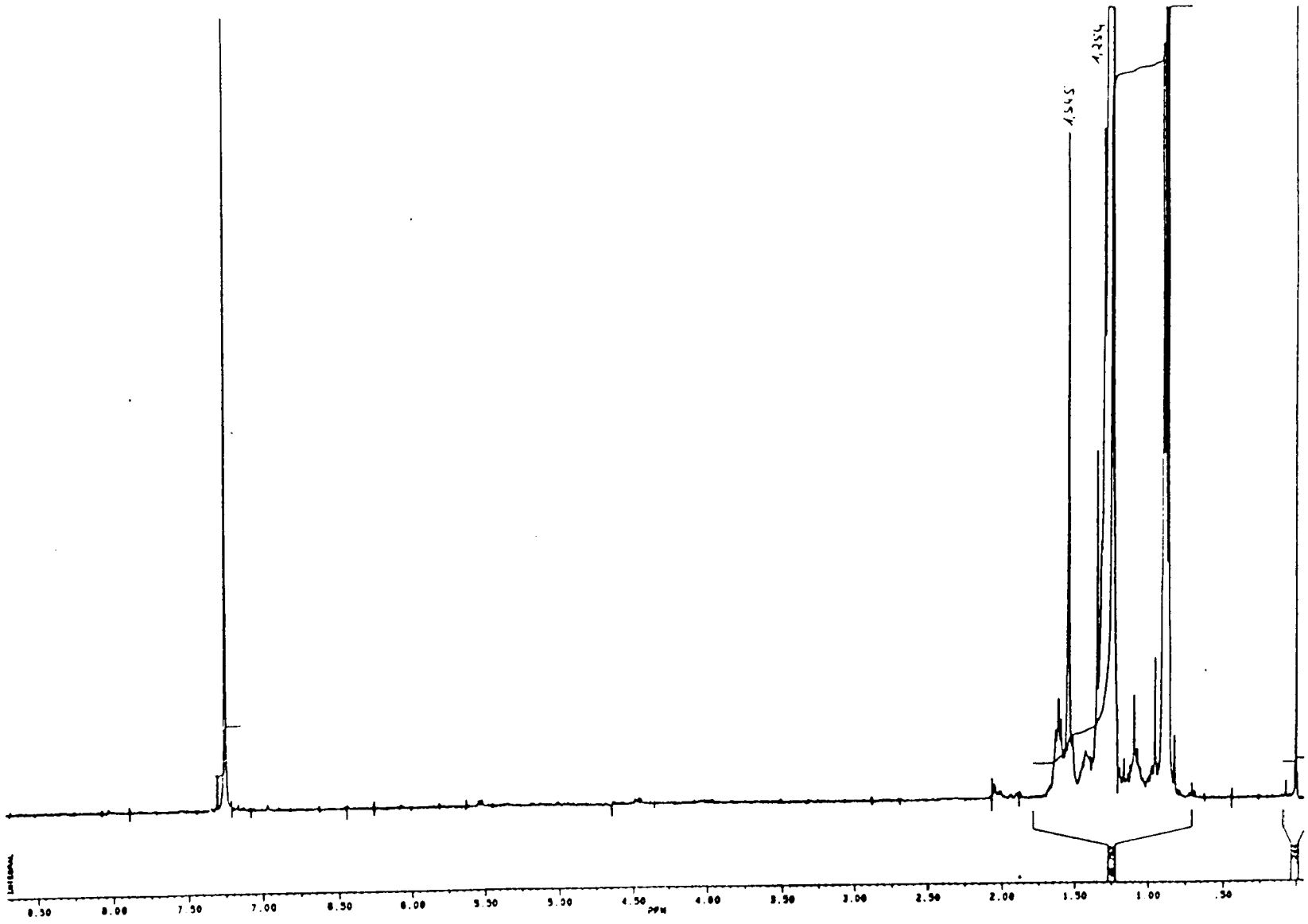


SPEC1: **EI-mass spectrum of hentriacontane [CH_3-$(CH_2)_{29}$-CH_3]**

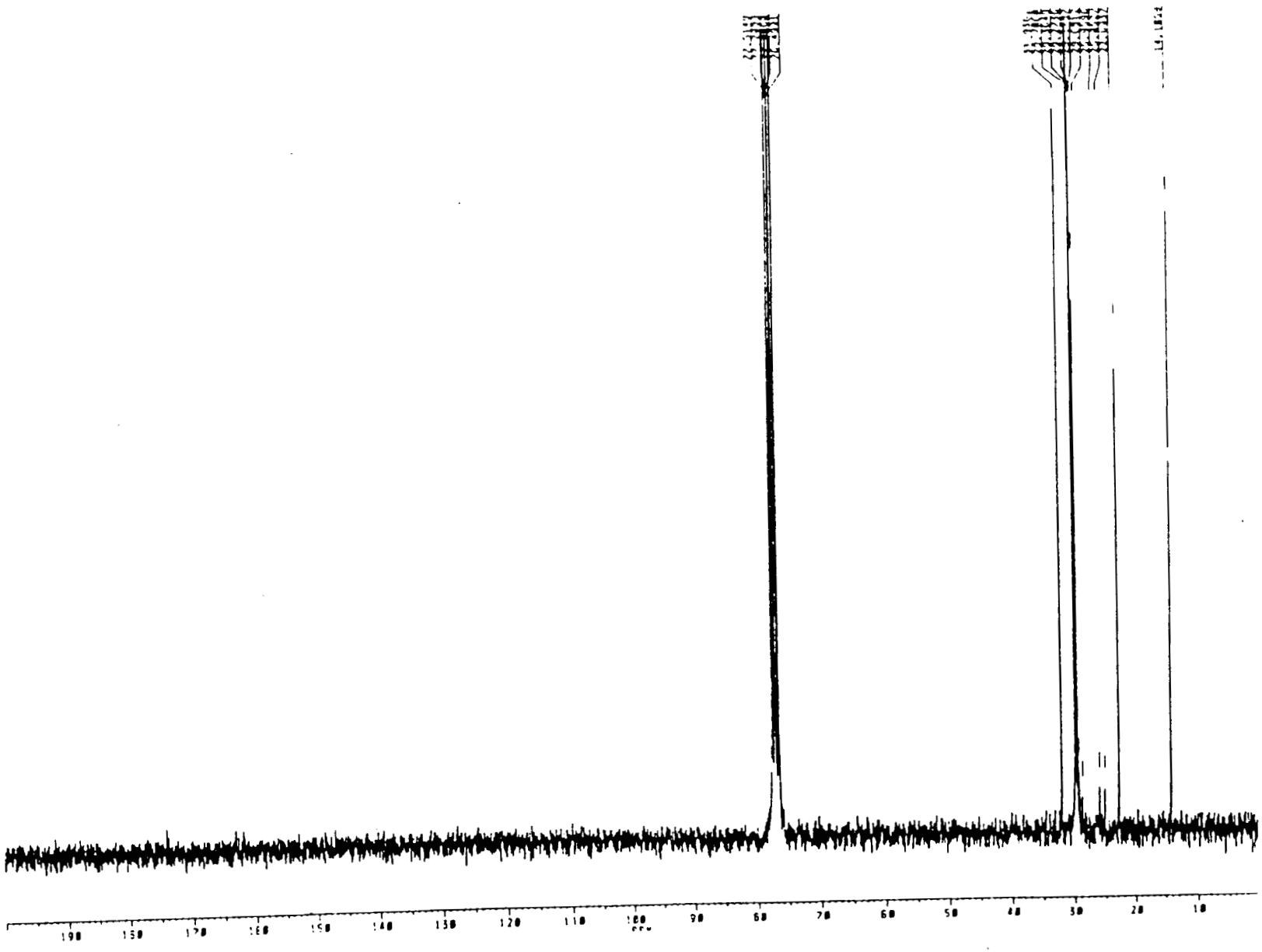


SPEC1: _

EI-mass spectrum of tritriacontane [CH_3-$(CH_2)_{31}$-CH_3]



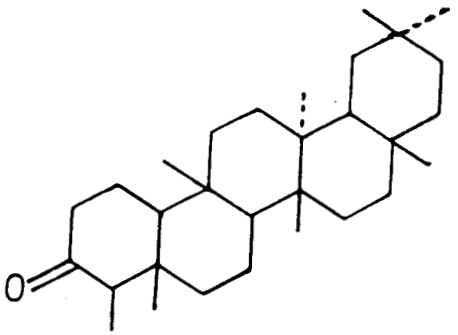
^{1}H-NMR spectrum of H_1 [mixture of alkanes] (360 MHz, $CDCl_3$, TMS)

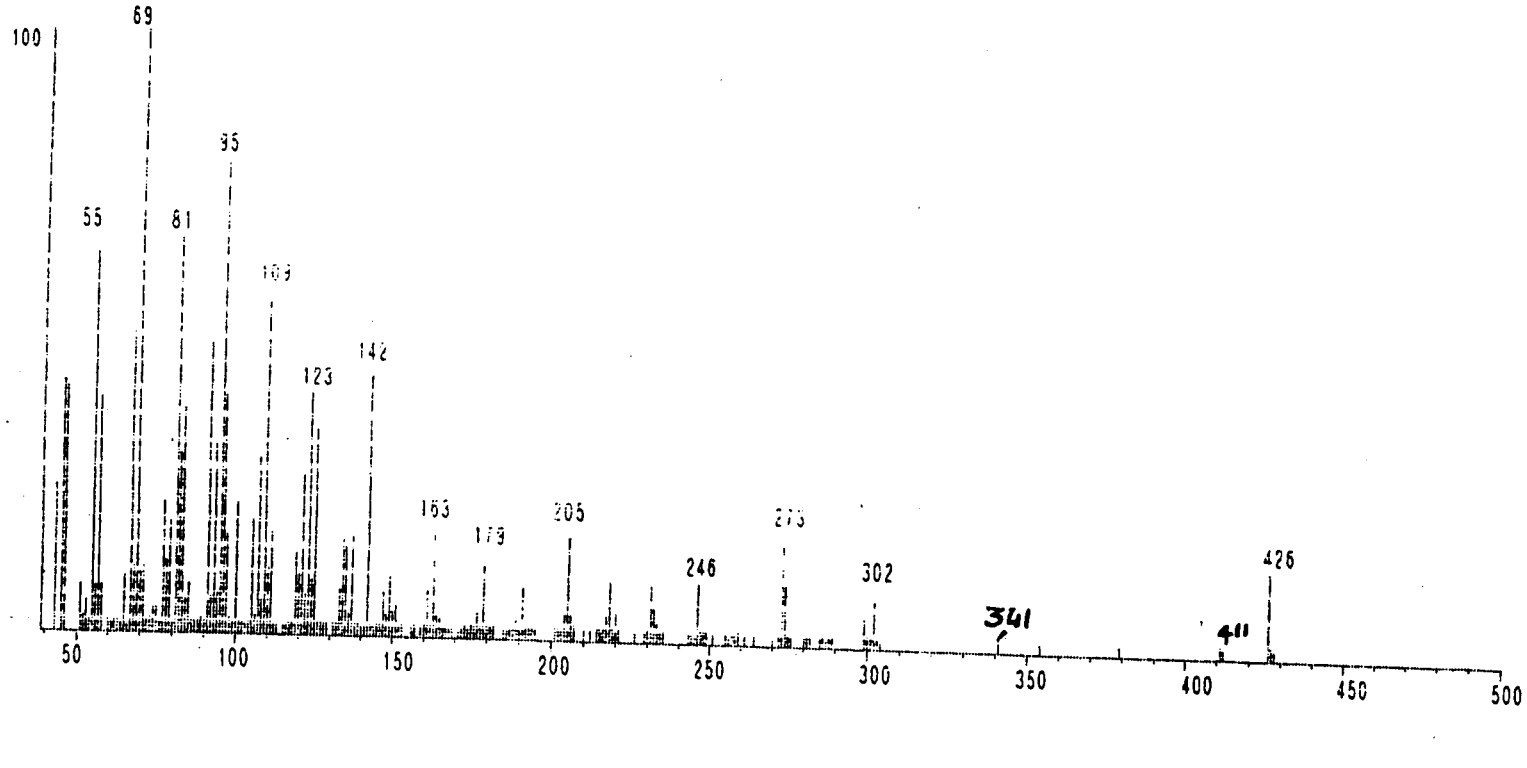


^{13}C NMR spectrum of H_1 [Mixture of alkanes] [63 MHz, $CDCl_3$, TMS)

I.4.2. Identification of H_2 (Friedelin)

Compound H_2 was isolated from the petroleum ether extract on elution with benzene and recrystallised from acetone as colourless crystalline needles (500 mg) which melted at 262°C. Compound H_2 answered Liebermann-Burchard colour reaction showing a persistent pink colour typical for triterpenes. EI Mass spectrum of the compound showed an M^+ ion at m/z 426. Fragmentation pattern with peaks at m/z 341, 302, 274 and 205 indicated it to be a friedelane type triterpenoid.[126] IR Spectrum showed a sharp peak at 1716.9 cm^{-1}, suggesting the presence of a C=O group. Other IR absorptions were at 2972.7, 2870.4, 1464.1, 1388.9, 1109.2 and 1072.6 cm^{-1}. Melting point, IR and mass spectral data were identical with that of friedelin.[127,128] Mixed m.p. with an authentic sample of friedelin was undepressed.

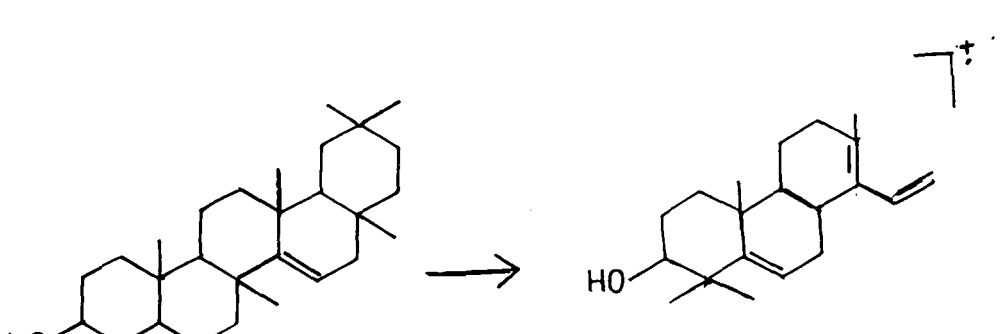




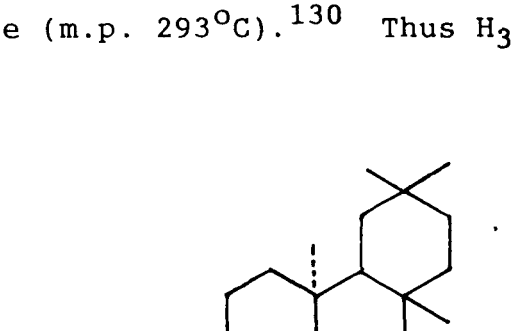
EI-mass spectrum of H_2 [Friedelin]

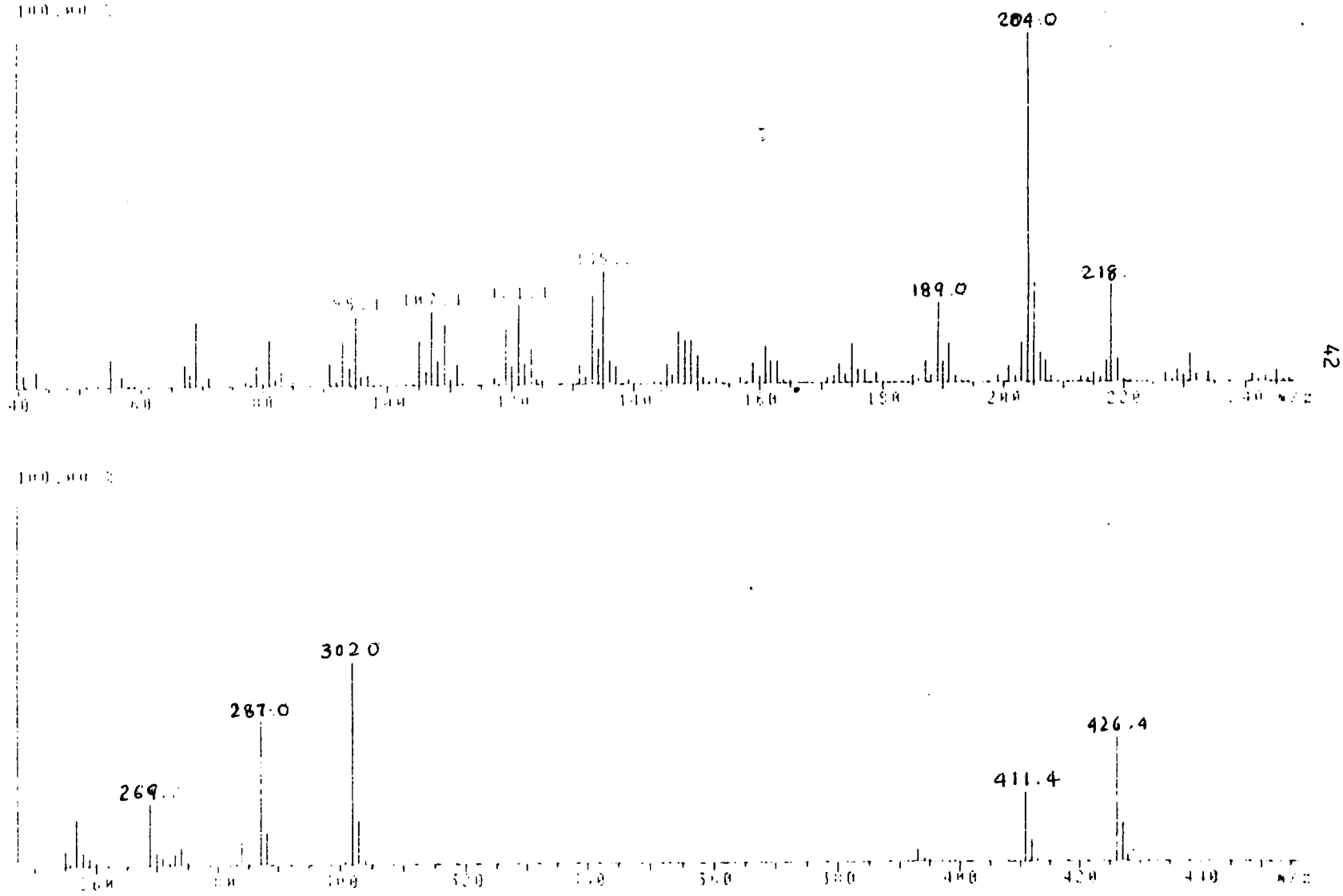
I.4.3. Identification of H_3 (Taraxerol)

This compound was isolated from the Petroleum ether extract on elution with 3:1 benzene-ethyl acetate as colourless needles (400 mg). It was recrystallised from benzene and had a m.p of 279°C. It answered Liebermann-Burchard colour reaction showing a persistent pink colour typical for triterpenes. High resolution mass spectrum of H_3 showed M^+ ion at m/z 426.4 corresponding to a molecular formula $C_{30}H_{50}O$. The IR spectrum of this compound showed a broad prominent absorption with the maximum at 3487.7 cm^{-1}, indicating the presence of a -OH group. The ^1H NMR spectrum showed eight tertiary methyl singlets at δ 0.80, 0.82, 0.91 (6H; 2 x Me), 0.93; 0.95; 0.98; and 1.09 indicating that it blongs to the pentacyclic group of triterpenes. The ^1H and ^{13}C NMR spectra showed the presence of a trisubstituted double bond [^1H NMR: δ 5.54 (1H, dd, vinylic proton); ^{13}C NMR: δ 158.17 (unsaturated quaternary carbon), 116.97 (unsaturated CH carbon)]. A multiplet at 3.2 in the ^1H NMR corresponding to one proton is that of a carbinol methine proton. This is supported by the presence of a carbinol methine carbon absorption at δ 79.1 in the ^{13}C NMR spectrum. These two observations along with the IR absorption for -OH group proved the presence of a secondary hydroxyl group. Prominent peaks in the mass spectrum were at m/z 426 (M^+), 411 (M^+-CH_3), 302, 287 (302-CH_3], 269, 218, 204 and 189.

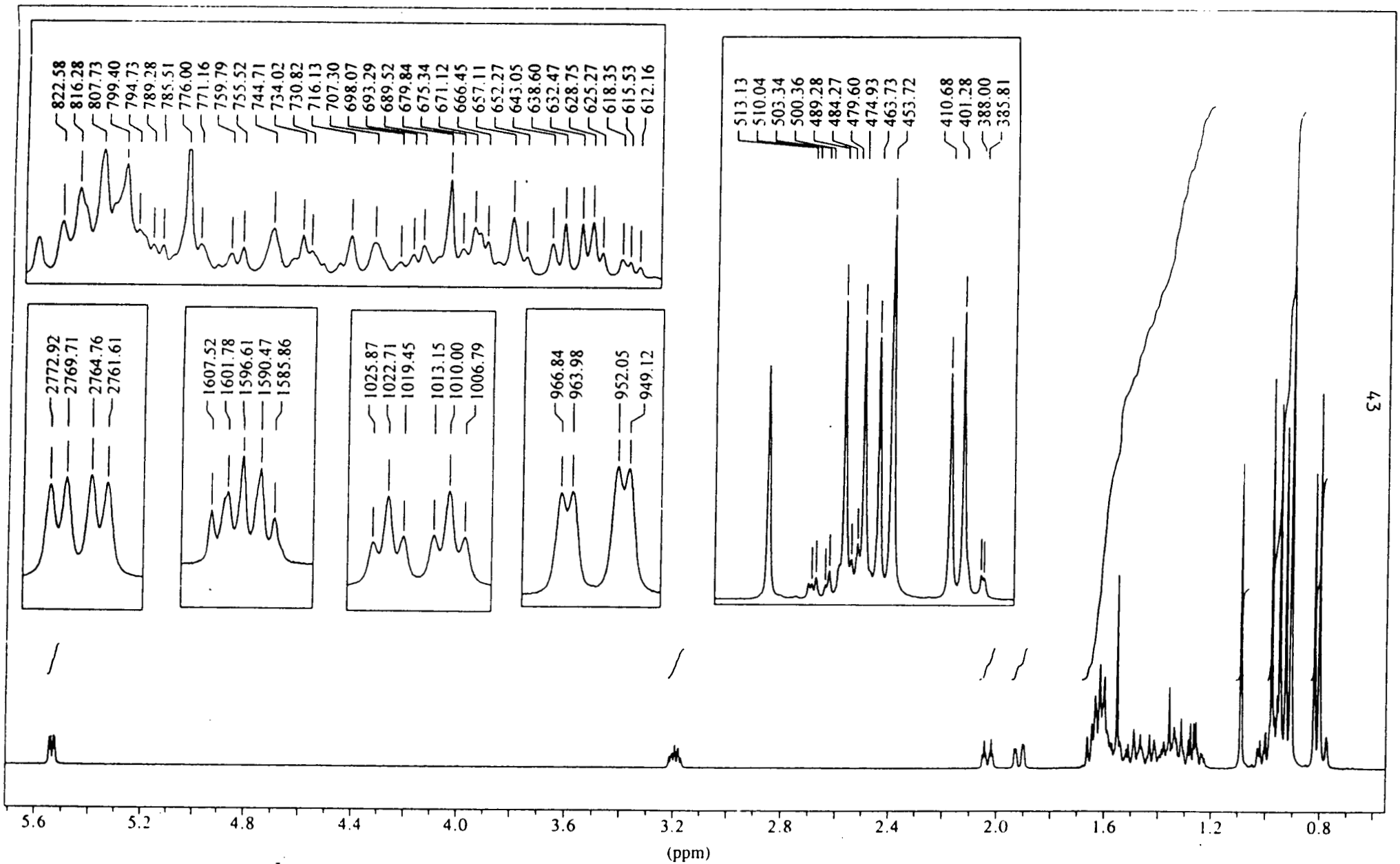


All the spectral data of compound H_3 were found to be quite identical with those of taraxerol reported in literature.[129] This compound on oxidation with Jones reagent gave the corresponding ketone melting at 242°C. This m.p. matched well with the reported m.p. (244°C) of taraxerone.[130] The acetate (m.p. 297°C) and benzoate (m.p. 289°C) of this compound were prepared and found to be identical with the reported m.p. of taraxerol acetate (m.p. 302°C) and benzoate (m.p. 293°C).[130] Thus H_3 was identified as taraxerol.

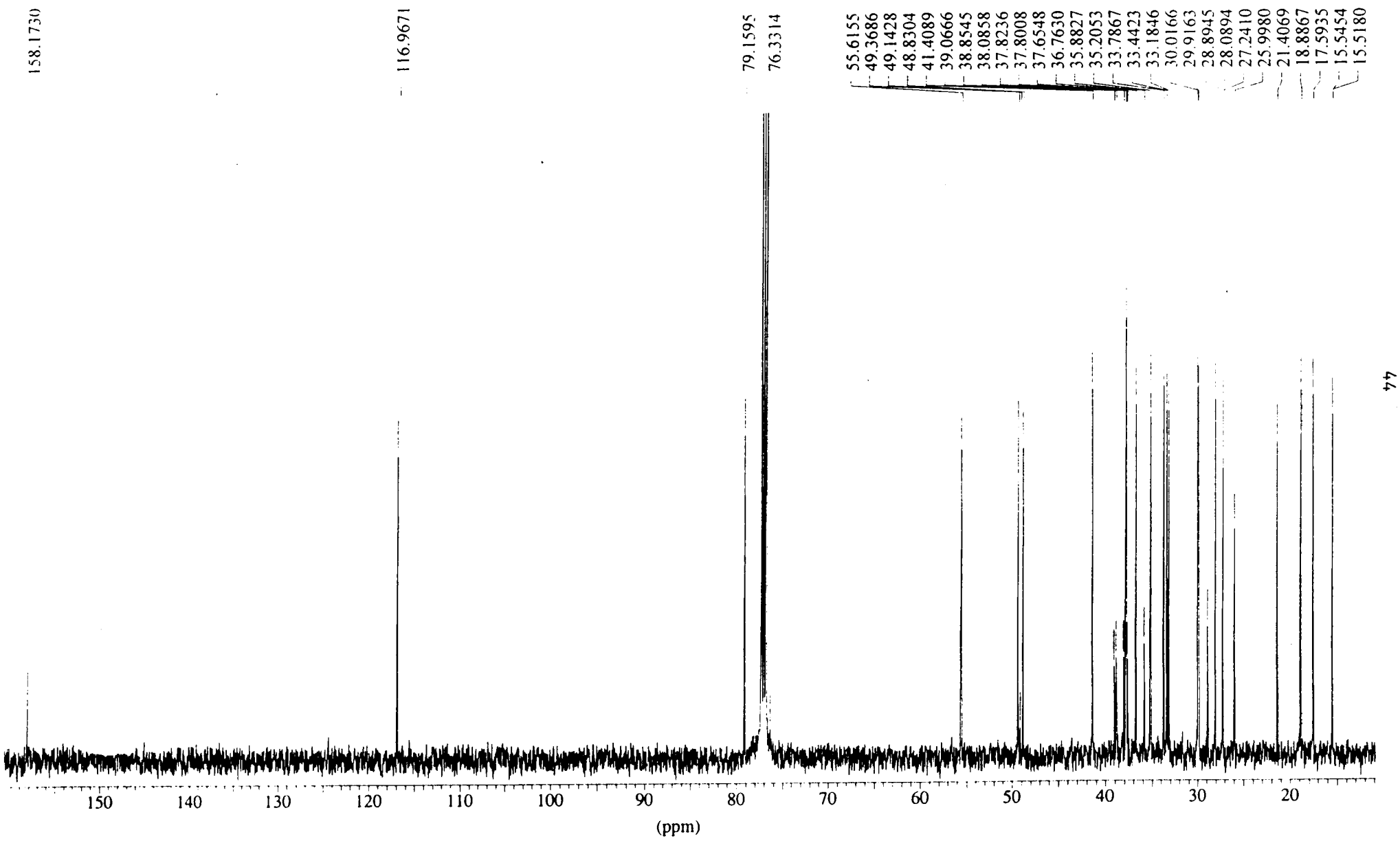




High resolution mass specturm of H_3 [Taraxerol]



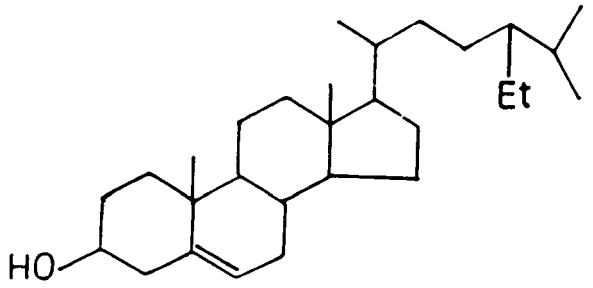
^{1}H-NMR spectrum of H_3 [Taraxerol] (500 MHz, $CDCl_3$, TMS)

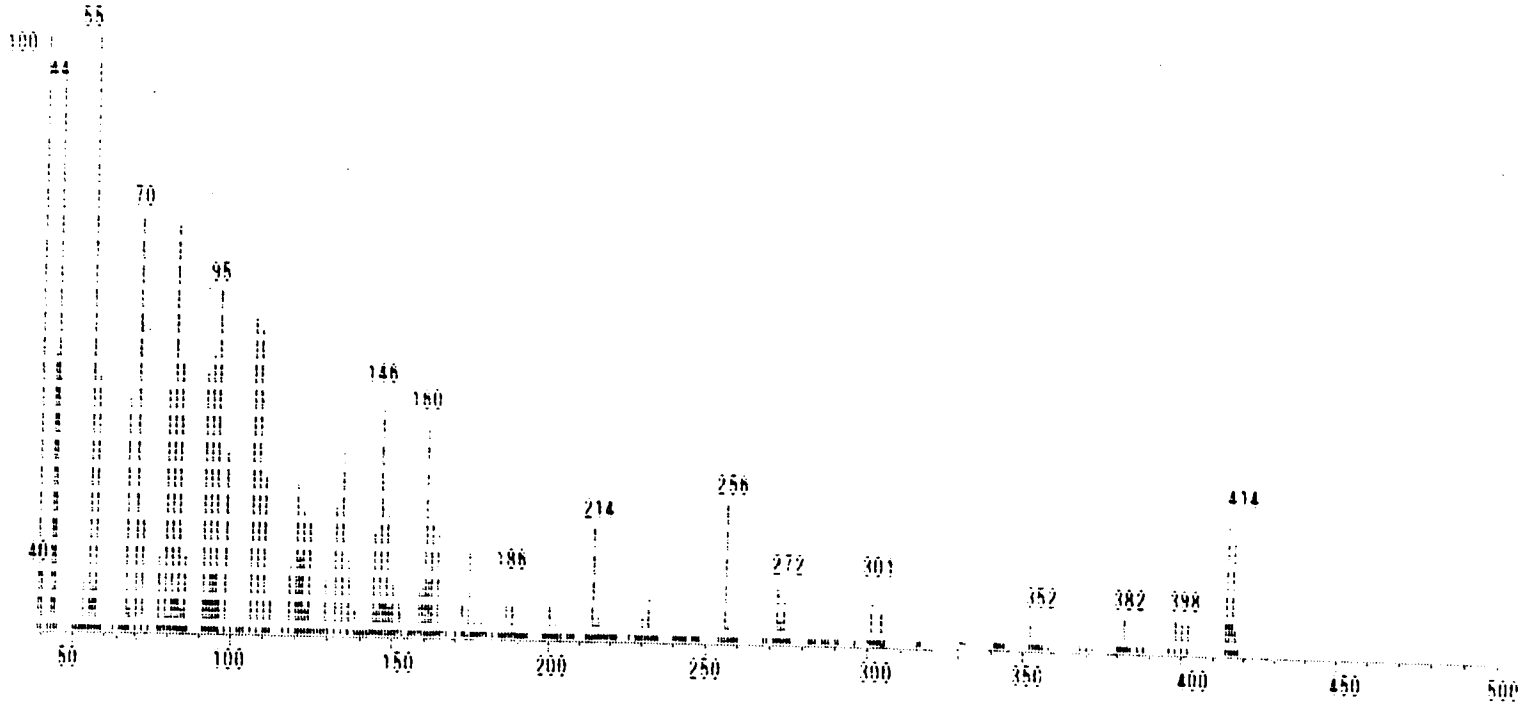


^{13}C-NMR spectrum of H_3 [Taraxerol] (125 MHz, $CDCl_3$, TMS)

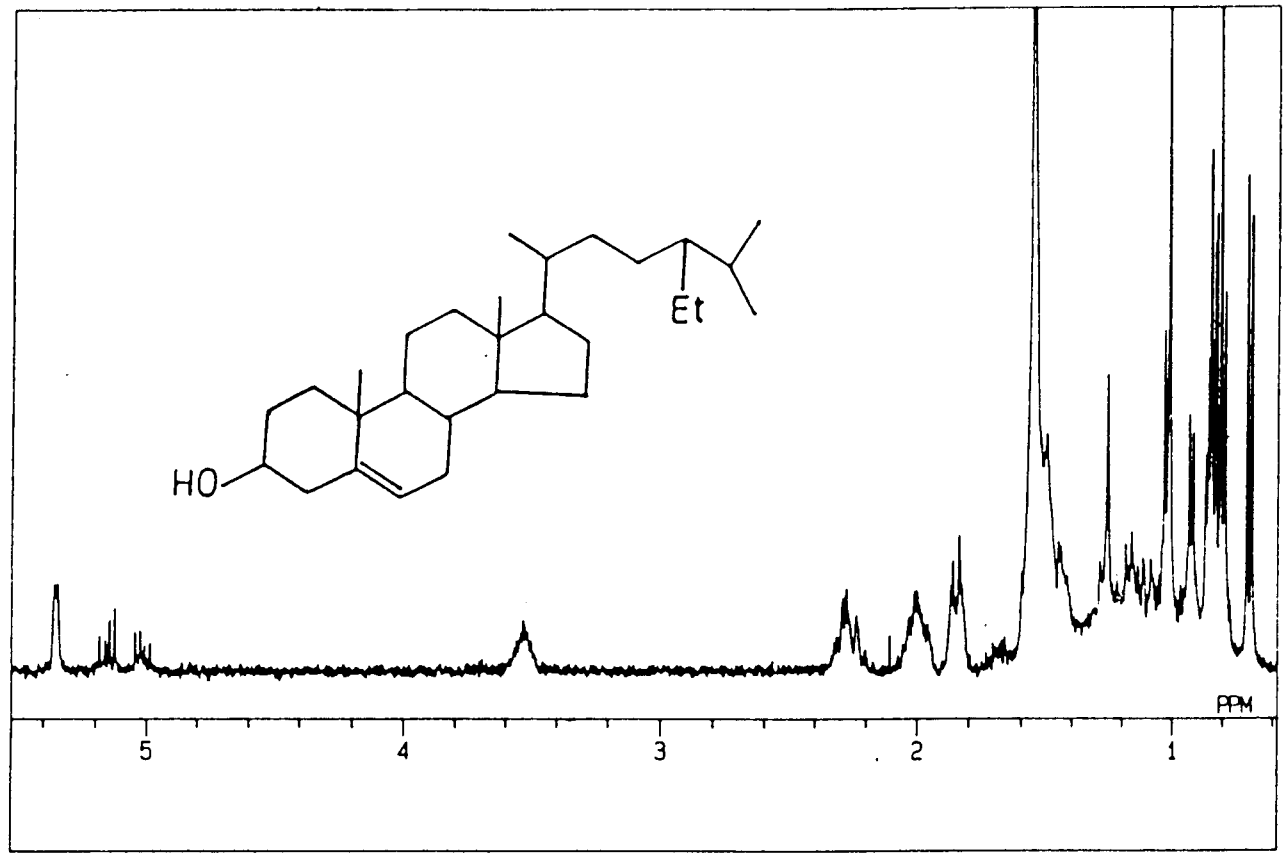
I.4.4. Identification of H_4 (β-Sitosterol)

This compound was isolated from the petroleum ether extract on elution with 3:1 benzene-ethylacetate as a white powdery substance. On crystallisation from methanol it yielded colourless needles (600 mg) melting at 139°C. With Liebermann-Burchard reagent it gave a play of colours indicating that it was a sterol. EI Mass spectrum of this compound showed M^+ at m/z 414 and base peak at m/z 55. IR Absorption spectra showed the presence of a hydroxyl group (broad absorption at 3441.4 cm^{-1}). Bands due to gem-dimethyl group [doublt at 1383.1 and 1385.1 cm^{-1}], -C-H stretching and bending bands [2981, 2816, 1464, 1470 cm^{-1}) were also observed in the spectrum, ^1H NMR Spectrum was quite comparable with that of β-sitosterol. A direct comparison of R_f with that of an authentic sample established its identity. Prepared the acetate (m.p. 125°C) and benzoate (m.p. 144°C) of this compound and was found to be identical with β-sitosterol acetate (m.p. 127°C) and benzoate (m.p. 145.5°C).[131]





EI-mass spectrum of H_4 [β-Sitosterol]

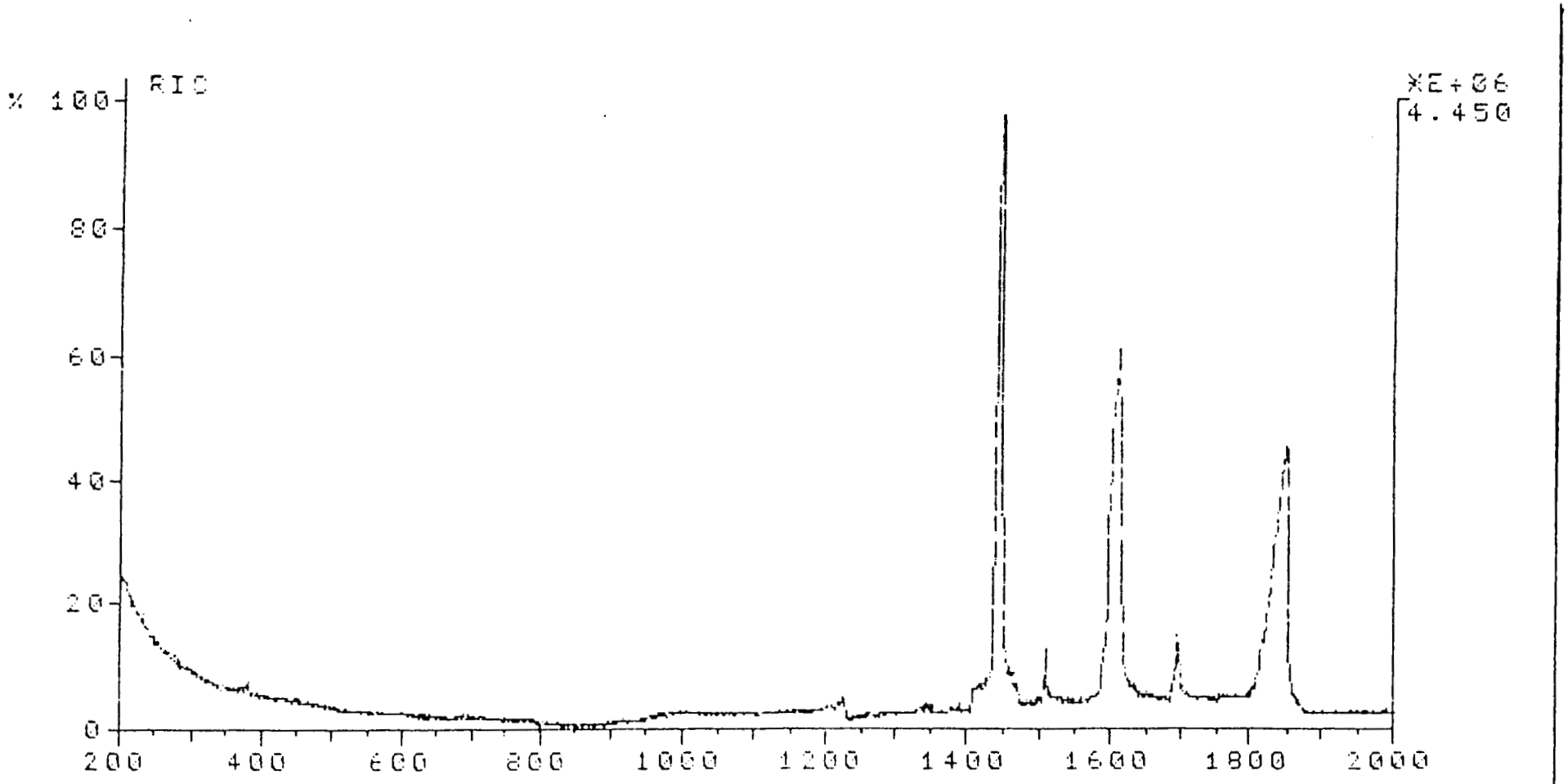


^{1}H-NMR spectrum of H_4 [β-Sitosterol](400MHz, $CDCl_3$, TMS)

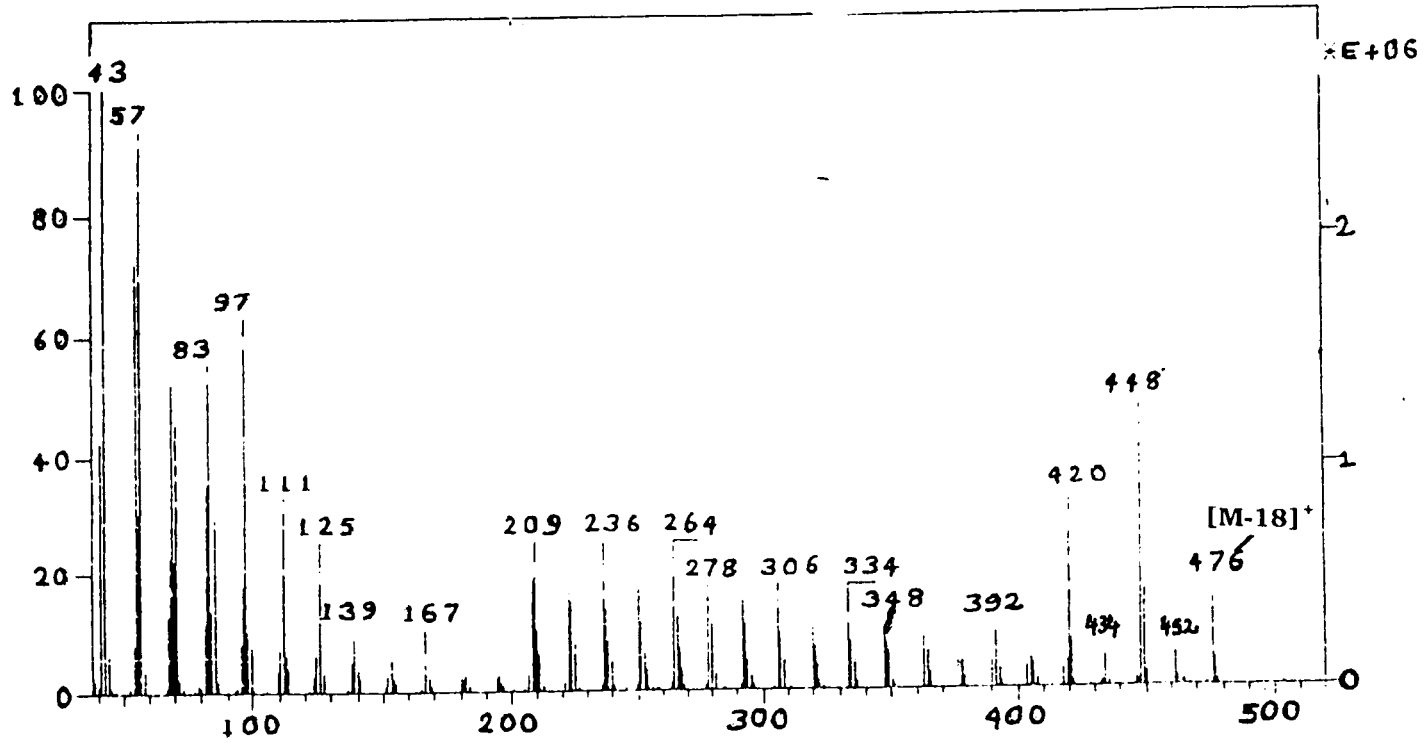
I.4.5. Identification of H_5

H_5 Was isolated from the petroleum ether extract on elution with 3:1 benzene-ethyl acetate as white powdery substance (650 mg), recrystallised from benzene and had a m.p. 82°C. TLC Analysis revealed the polar nature of the component. It didn't answer Liebermann-Burchard test. IR Spectrum showed the presence of a hydroxyl group (Broad absorpiton at 3450 cm^{-1}). Other prominent absorbtion bands were at 2918.7, 2849.2 (C-H stretching), 1478, 1462.2 (-C-H bending), 1061.9 (C-O-stretching) 734.1 and 719 cm^{-1} (long chain aliphatic compound). ^1H NMR spectrum (absorptions in the region δ 0.9-1.6) and ^{13}C NMR spectrum (absorptions at δ14.1, 22.65, 25.72, a number of absorptions around 29 and the absorptions at 31.92, 32.31) indicated a long hydrocarbon chain. A triplet at δ 3.63 in the ^1H NMR Spectrum corresponding to two protons and an absorption at δ63.11 in the ^{13}C NMR indicated the presence of $-CH_2-OH$ group in the compound. EI Mass spectrum of H_5 showed the base peak at m/z 57 and the maximum m/z value at 476. The high intensity of peaks at m/z 448, 420, were suggestive of a mixture of compounds. This doubt could be resolved by conducting GC-MS analysis of H_5. In the GC-MS analysis the temperature was programmed from 60° to 320° at a rate of 15°C/minute and held at 320°C for 15 minutes. Helium was used as the carrier gas. GC Analysis indicated that the

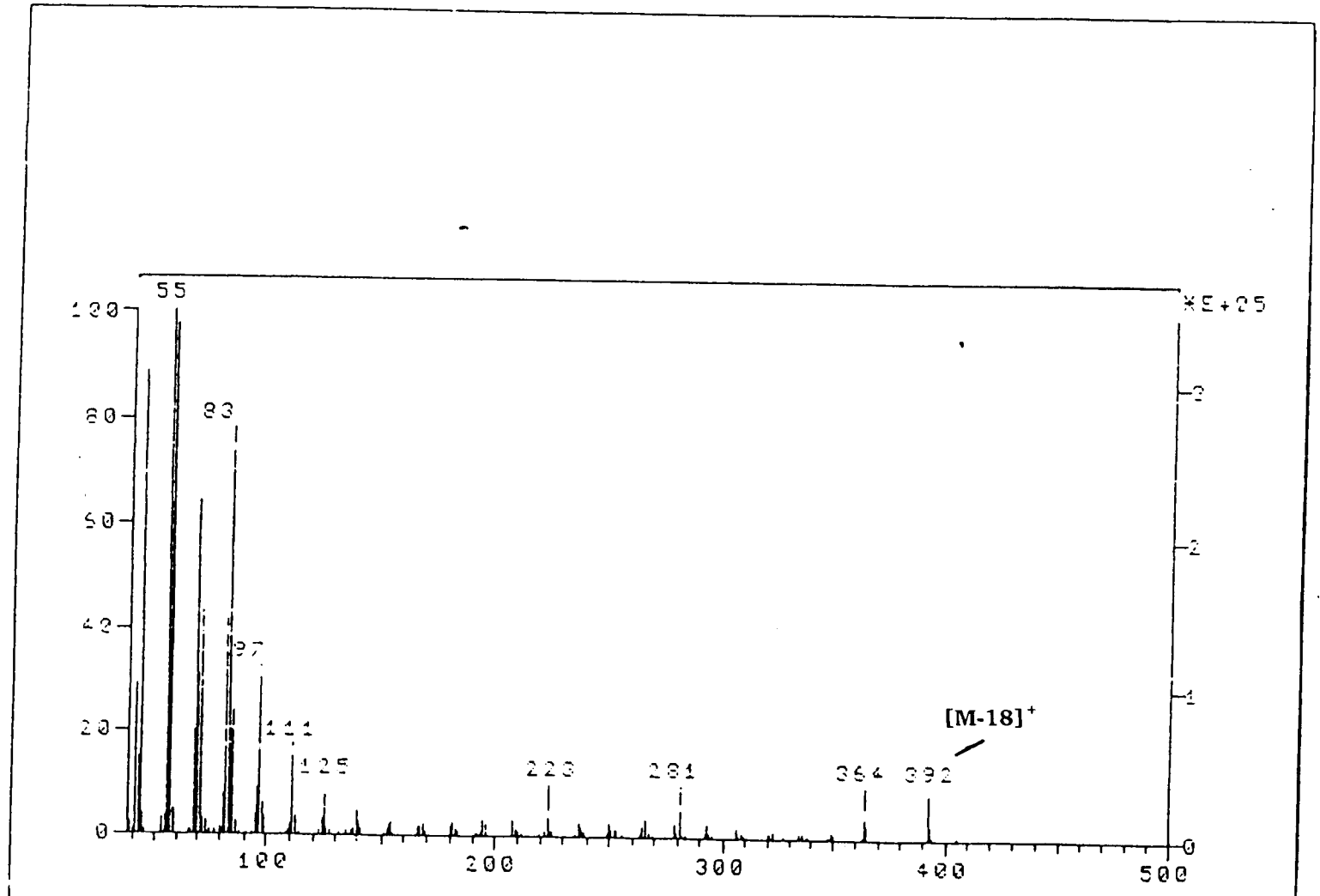
component H_5 was a mixture. Mass spectrum did not show any molecular ions but characteristic ions at $[M-18]^+$, due to elimination of water. Mass spectra of octacosanol, dotriacontanol and tritriacontanol are presented. From the retention times, GC-MS analysis, IR and NMR data H_5 was identified as a mixture of long chain 1-alkanols, the major component being dotriacontanol, $CH_3-(CH_2)_{30}-CH_2OH$ (about 45% of the total mixture]. Other major components were triacontanol, $CH_3(CH_2)_{28}-CH_2OH$ (about 25%), tetratriacontanol, $CH_3-(CH_2)_{32}-CH_2OH$ (about 15%) and octacosanol, $CH_3-(CH_2)_{26}-CH_2OH$ (about 8%) together with minor components of this series $CH_3-(CH_2)_{27}-CH_2OH$ (nonacosanol), $CH_3-(CH_2)_{29}-CH_2OH$ (hentriacontanol) and $CH_3-(CH_2)_{31}-CH_2OH$ (tritriacontanol). The concentrations given are peak area percentage obtained from GC. Peaks corresponding to tritriacontanol and tetratriacontanol are not shown in the GC plot.



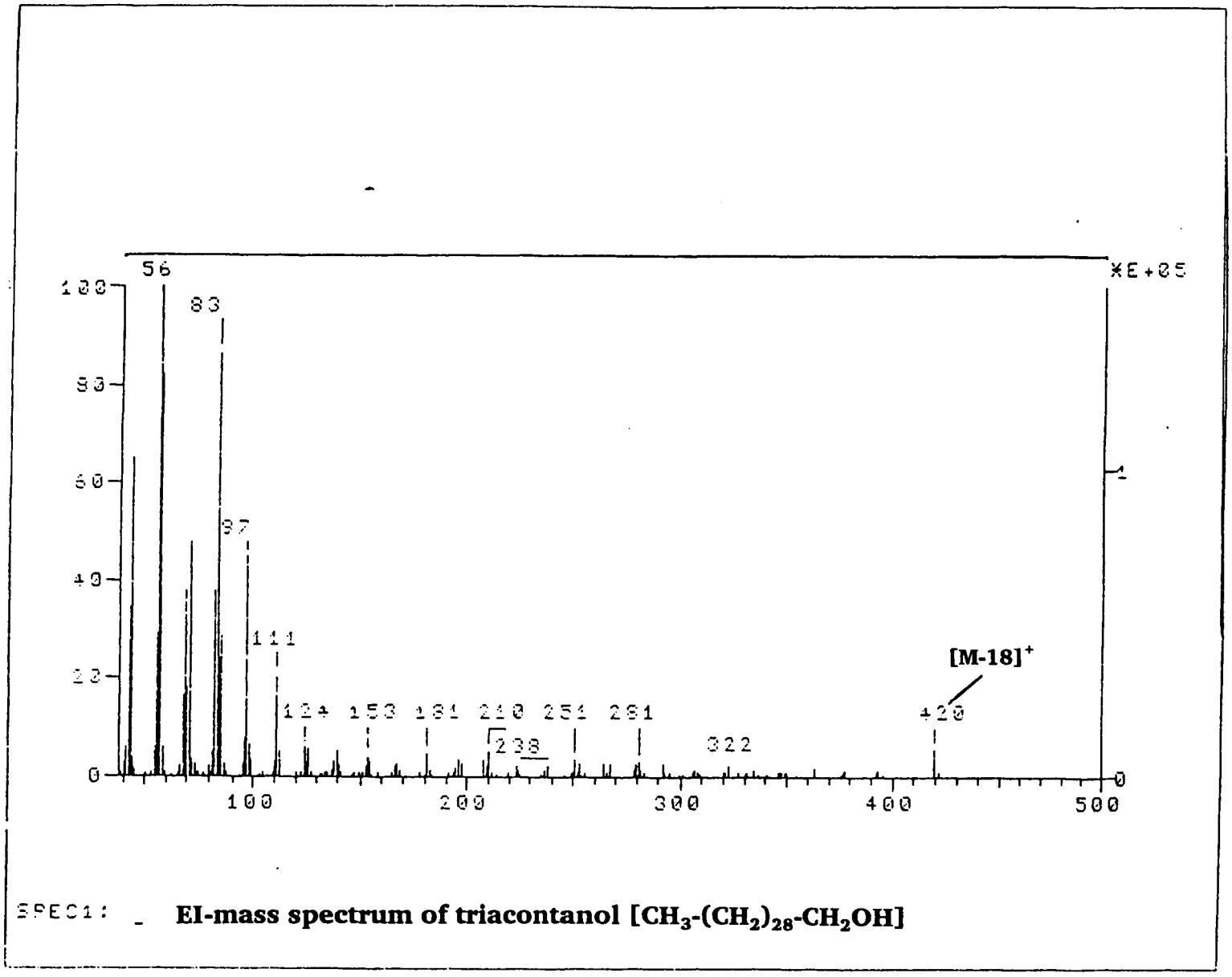
Gas chromatogram of H_5 [Mixture of alkanols]



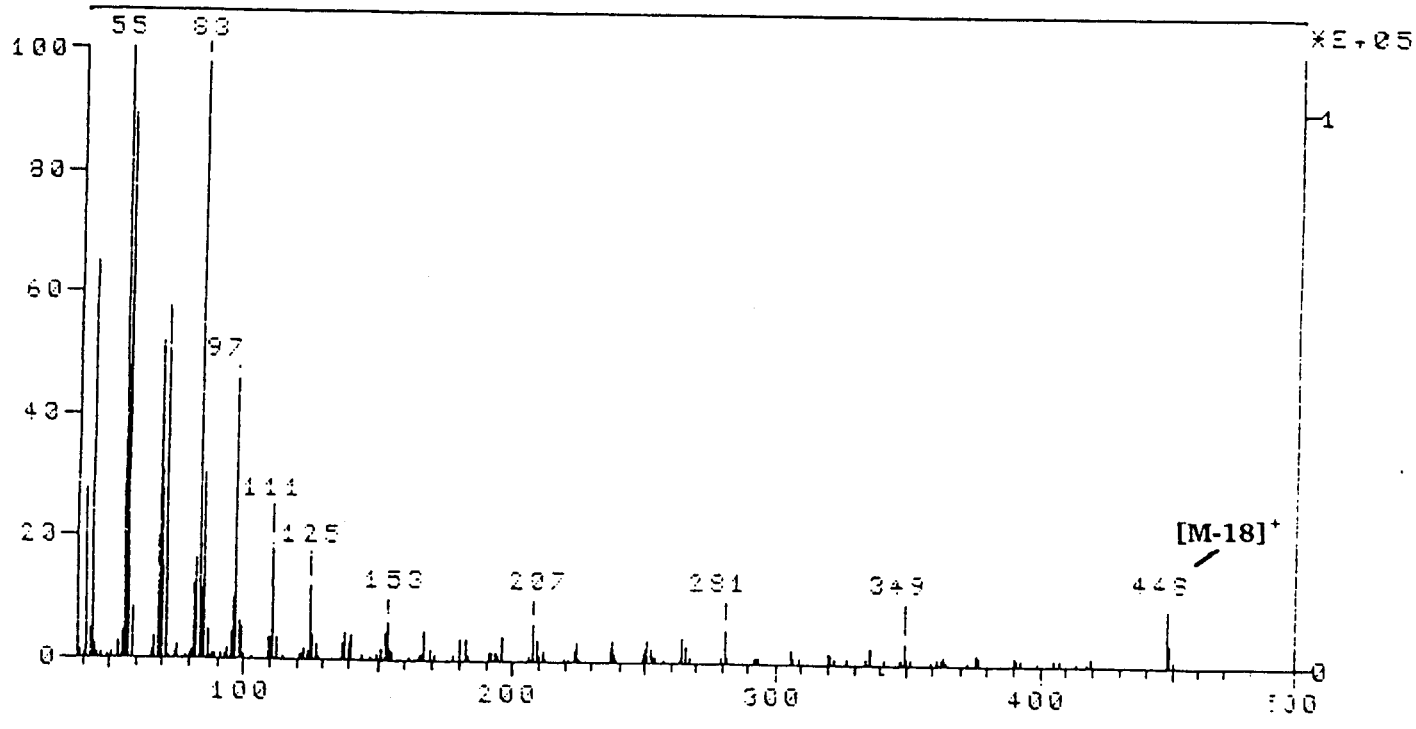
EI-mass spectrum of H_5 [Mixture of alkanols]



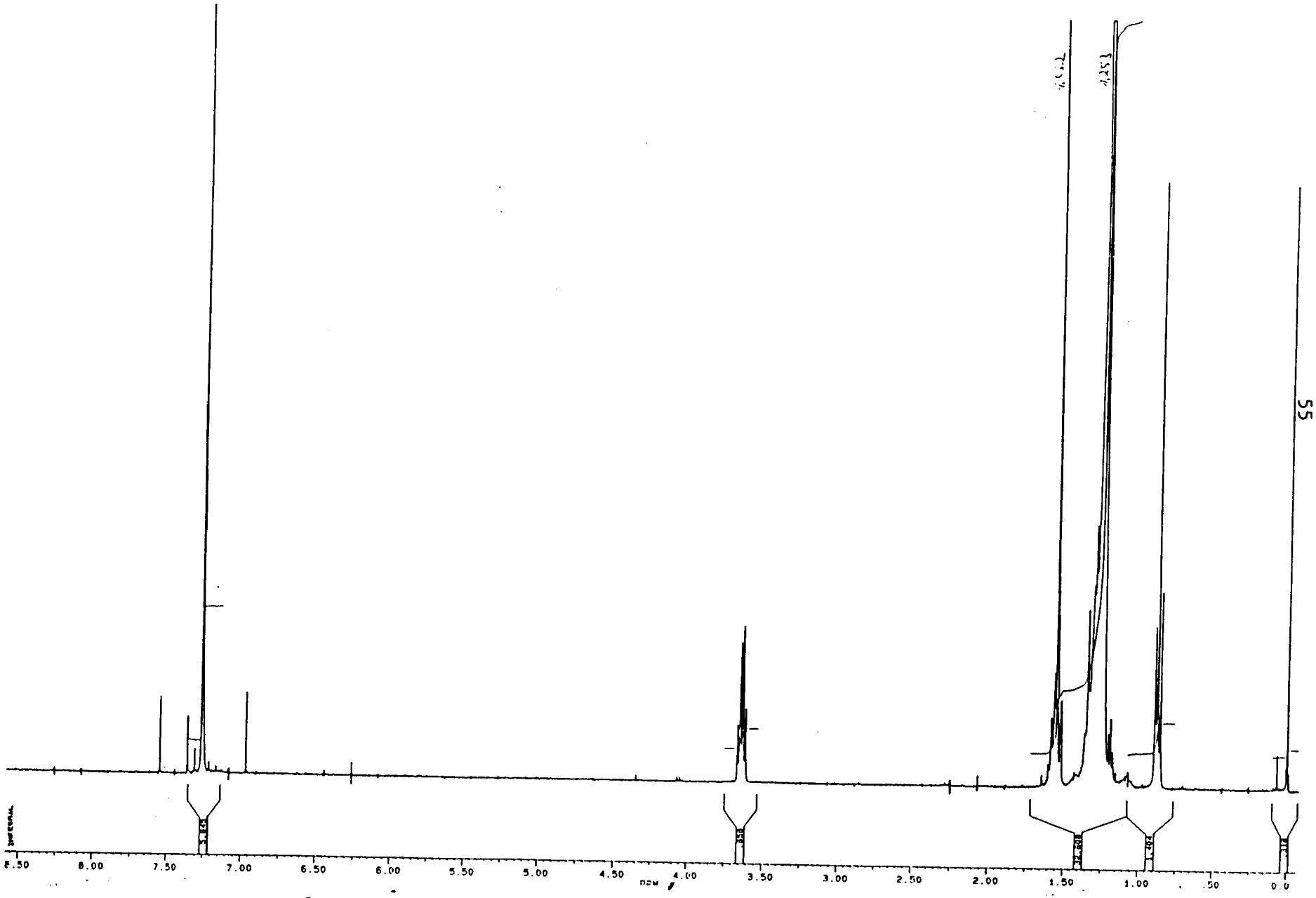
SPEC1: **EI-mass spectrum of Octacosanol [CH_3-$(CH_2)_{26}$-CH_2OH]**



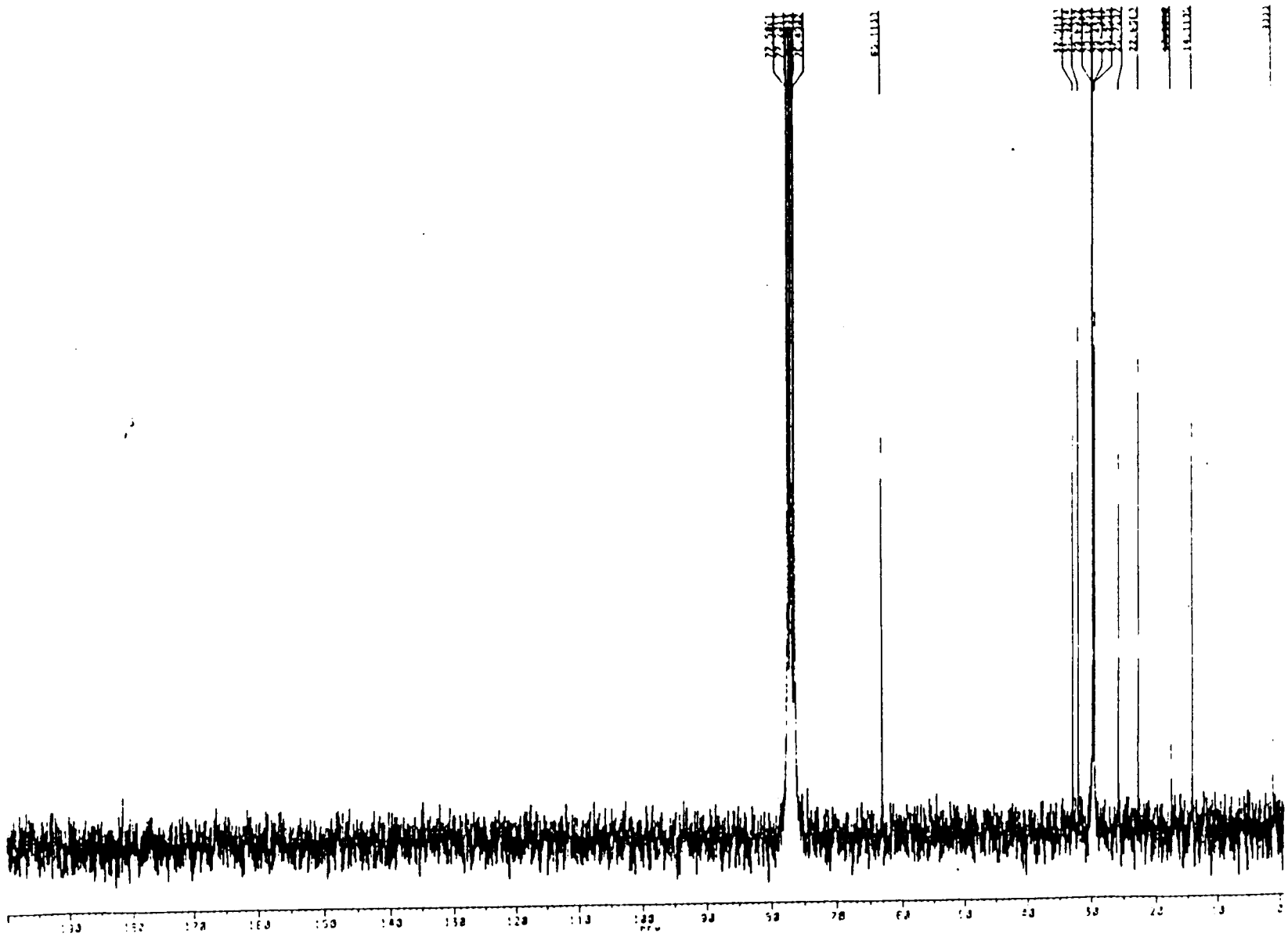
EI-mass spectrum of triacontanol [CH_3-$(CH_2)_{28}$-CH_2OH]



SPEC1: **EI-mass spectrum of dotriacontanol [CH_3-$(CH_2)_{30}$-CH_2OH]**



^{1}H-NMR spectrum of H_5 [Mixture of alkanols] (360MHz, $CDCl_3$, TMS)



^{13}C-NMR spectrum of H_5 [Mixture of alkanols] (63 MHz, $CDCl_3$, TMS)

I.5. EXPERIMENTAL

I.5.1. Jones oxidation of the compound H_3, (Taraxerol)

Anhydrous CrO_3 (2.67 g) was dissolved in 2.3 mL concentrated H_2SO_4 and diluted with 15 mL of water. Compound H_3 (50 mg) was dissolved in 20 mL acetone and cooled in an ice bath. Jones reagent (0.5 mL) was added to the cold solution with constant stirring. The reaction mixture was kept stirring for 5 minutes. Excess chromic acid was decomposed by adding sodium sulphite followed by K_2CO_3. The product was extracted with ether, washed with K_2CO_3 solution and with water. The extract after drying over anhydrous sodium sulphate was evaporated to dryness, which on purification by preparative TLC and recrystallisation from petroleum spirit yielded shining crystals melting at 242°C. IR Spectrum of this compound showed a sharp peak at 1736.1 cm^{-1}.

I.5.2. Acetylation of H_3 (Taraxerol)

100 mg of H_3 was refluxed with 3 mL pyridine and 1 mL acetic anhydride for about 2 h. The reaction mixture was poured into cold water, filtered, washed with water and then recrystallised from benzene. The product melted at 297°C.

I.5.3. Acetylation of H_4 (β -Sitosterol)

100 mg of H_4 was refluxed with 3 mL pyridine and 1 mL acetic anhydride for about 2 h. Poured the reaction mixture to cold water, filtered, washed with water and then recrystallised from petroleum spirit. The product melted at 133°C.

I.5.4. Benzoylation of H_3 (Taraxerol)

Benzoylation of H_3 was done by refluxing the compound (100 mg) with freshly distilled benzoyl chloride (2 mL) and pyridine (5 mL) on a sand bath for 2 h. The reaction mixture was then poured into cold water, filtered the precipitate, washed with water and recrystallised from benzene. The benzoylated product melted at 289°C.

I.5.5. Benzoylation of H_4 (β-Sitosterol)

100 mg of H_4 was refluxed with 2 mL freshly distilled benzoyl chloride and 5 mL pyridine on a sand bath for 2 h. Poured the reaction mixture into cold water, filtered, washed with water and recrystallised from benzene. The product of benzoylation of H_4 melted at 144°C.

SECTION 1: THE PHYTOCHEMISTRY OF ANNONACEAE - A REVIEW

SECTION 2 : PHYTOCHEMICAL STUDIES ON THE LEAVES OF UVARIA NARUM

Bindu. T. K "Phytochemical investigation of a few plants" Thesis. Department of Chemistry, University of Calicut, 1998

CHAPTER II

SECTION 1: THE PHYTOCHEMISTRY OF ANNONACEAE - A REVIEW

Introduction

The Annonaceae family is very large, comprising about 120 genera and more than 2000 species. This family has appreciable economic importance as a source of edible fruits and oils. Many members like Paw Paw (Asimina triloba), Annona cherimolia, Annona squamosa, Annona muricata and some members of the genera Cananga and Rollinia are cultivated in many countries for their edible fruits. Oils from the seeds of some plants like A. muricata, A. senegalensis may be used for the production of soap and as edible oils.[132] Flowers of some species like Cananga odorata are used in perfumery and many members of this family are used in the folk medicine of several countries for various purposes. Some members of Annonaceae have been described as cytotoxic and they are used in folk medicine of some tropical countries to treat various tumors and cancers.

Chemical and pharmacological studies on Annonaceae plants have been intensified only in the last decade. According to Panichpol and Waterman, 'for its size, the

Annonaceae is perhaps one of the least chemically known families'.[133] Most investigations have centered upon alkaloids, but Annonaceae also produce a wide range of non-alkaloidal compounds belonging to various phytochemical groups.

The chemical and pharmacological features of some groups of compounds isolated from Annonaceae tempts chemists and pharmacologists to investigate further on these plants. A comprehensive review article published by Leboeuf et al. in 1982 updates all the important phytochemical research on Annonaceae members.[134] Recently, there are a large number of reports on the isolation, identification, and biological studies of a group of extremely bioactive compounds, called Annonaceous acetogenins.[135]

Distribution

The Annonaceae family consists of aromatic trees, shrubs, climbers, usually evergreen, which grow in tropical and subtropical areas. About 450 species belonging to 51 genera are growing in Asia and Australia. About 950 species are growing in Africa and Madagascar. American continent contains 740 species belonging to 38 genera. Asia together

with Australia is the basic centre of distribution of Annonaceae plants.

Classification

The Annonaceae members are characterized by a great many primitive and archaic features. They are what Darwin called 'living fossils', which through some favourable circumstances have escaped extinction and survived to the present. Annonaceae are included within the Magnoliales (Annonales) with the most primitive families of angiosperms, but they are noticeably advanced. Annonaceae family is notoriously difficult to divide into natural subgroupings of genera.

Non Alkaloidal Components of Annonaceae

Carbohydrates, Sugars, Amino acids and Proteins.

A lot of studies have been done on the sugars, carbohydrates amino acids and protein content of seeds and fruits of many Annonaceae members, due to their nutritional and economic importance. The fruits and seeds of Annona cherimolia, Annona reticulata, Annona senegalensis, Annona squamosa and Annona muricata were studied for the above constituents.[134]

Lipids

Oils and fatty acids have been isolated from the seeds and fruits of many Annonaceae members. Leaf wax of some members like Annona senegalensis, was found to contain saturated and unsaturated fatty acids and primary alcohols of C_{28}, C_{30} and C_{32}.[134] Studies on the various fatty acids isolated from the seeds and fruits of some members like Annona cherimolia, Annona muricata, Annona squamosa, Cananga odorata etc. shows that they are semidrying oils containing various proportions of oleic and linoleic acids and saturated acids like myristic, palmitic and stearic acids. Other unsaturated acids like palmitoleic acid and linolenic acid exsists only in small amounts.[134] Glycerides of one or more hydroxylated fattyacids of unusually high molecular weight possessing toxic and insecticidal properties have been reported from the seeds of several species of Annona, especially A. reticulata before 1982. These peculiar compounds were later charecterized as a new group of natural products, namely tetrahydrofuranoid acetogenins or Annonaceous acetogenins. Recently a lot of research work is being done to isolate and characterize these compounds having extremely high bio-activities.[135]

Polyphenols and Flavonoids

A number of polyphenolic compounds are isolated from many members of Annonaceae. Phenolic acids like caffeic acid, p-hydroxybenzoic acid, p-coumaric acid, vanillic acid etc. are elaborated by many members of this family. Catechins, proanthocyanidins and tannins are also isolated from some members. The common flavonoids like quercetin, quercitrin, rutin etc. are present in the leaves of many members. In addition to this, many memebers of this family are reported to contain acylated and methylated flavonoids.

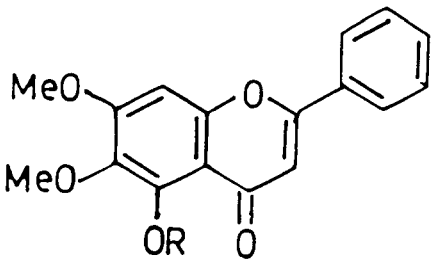
Recently, a large number of flavonoids, like 5,6,7-trimethoxyflavone [1], 5-hydroxy-6,7-dimethoxyflavone [2], 5,7,8-trimethoxyflavanone [3], 2'-hydroxy-3',4',6'-trimethoxychalcone [4], 2',3',4',6'-tetramethoxychalcone [5], 2',4-dihydroxy-3',4',6'-trimethoxychalcone [6], 5,6,7,8-tetramethoxyflavanone [7] and 2'-hydroxy-3',4',5',6'-tetramethoxychalcone [8] are isolated from the stem and ripe fruits of one member of this family, Monanthotaxis cauliflora[134]. A common feature among these flavonoids, except [6], is the absence of B-ring substitution, a trait which may prove to have taxonomic significance. Another point is the change from C-8 to C-6 substitution in the transition from chalcone or flavanone to

flavone. According to Harborne, C-8 hydroxylation is characteristic of primitive families.[133]

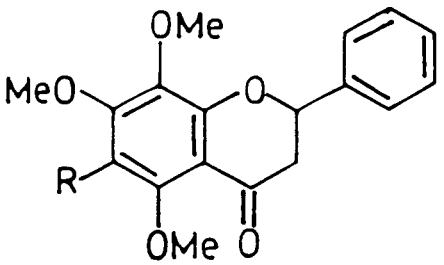
Since 1976, flavonoids of a special type have been obtained from several Uvaria species. These are the novel C-benzylated flavanones and C-benzylated dihydrochalcones derived from the known flavanone, Pinocembrin [9]. Some of them have demonstrated cytotoxic, antitumor and antimicrobial properties. Although flavanones and chalcones are wide-spread in higher plants, the introduction of benzyl groups is quite rare and seems to be limited to Uvaria. Absence of substituents on the B-ring in all flavonoids of Uvaria can be linked with the previous observation concerning the flavonoids of Monanthotaxis cauliflora.[133] The flavonoids isolated from Uvaria species are listed in the Table II. 1.

Table II.1 **The flavonoids isolated from Uvaria species**

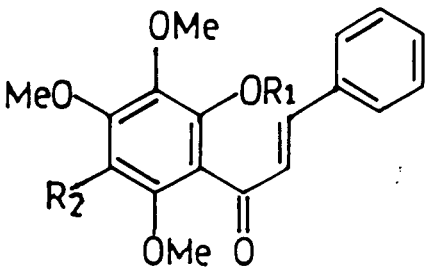
Name	Structure	Species	Reference
Pinocembrin	9	U. chamae	136-138
Pinostrobin	10	U. chamae	138
Uvangoletin	11	U. angolensis	139
Angoletin	12	U. angolensis	139
Chamanetin	13	U. chamae	136-138, 140,141
Chamanetin 5-methylether	14	U. chamae	142,143
Isochamanetin	15	U. chamae	136-138, 140,141
Uvaretin	16	U. acuminata	144,
		U. chamae	136,138 145,146
		U. angolensis	139
		U. kirki	147
Isouvaretin	17	U. angolensis	139
		U. chamae	136-138
Dichamanetin	18	U. chamae	142,143
Dichamanetin-5-methylether	19	U. chamae	136,138
Diuvaretin	20	U. chamae	145
Chamuvaritin	21	U. chamae	136,138 148,149
Uvarinol	22	U. chamae	136,138 148,149
Vafzelin	23	U. afzelii	150
Uvafzelin	24	U. afzelii	150
Syncarpic acid	25	U. afzelii	150



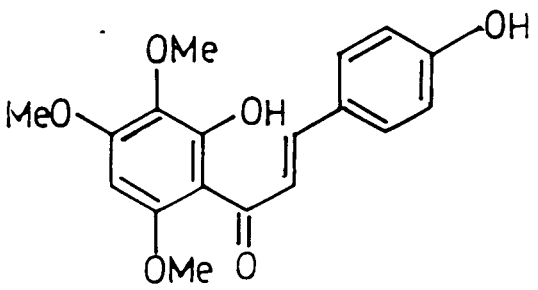
[1] R = Me
[2] R = H



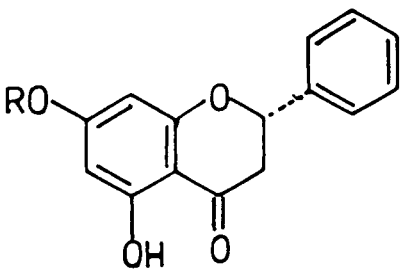
[3] R = H
[7] R = OMe



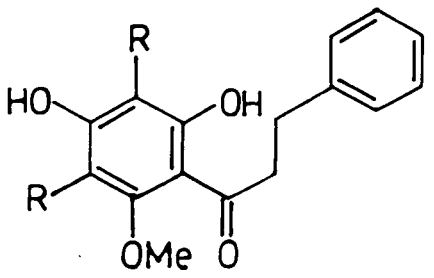
[4] $R_1 = R_2 = H$
[5] $R_1 = Me$, $R_2 = H$
[8] $R_1 = H$, $R_2 = OMe$



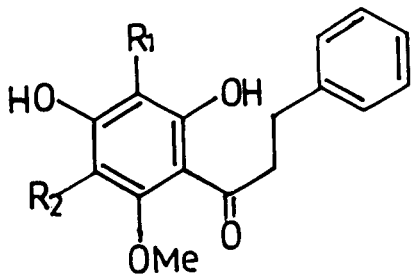
[6]

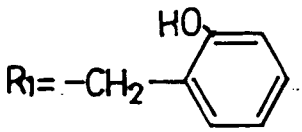


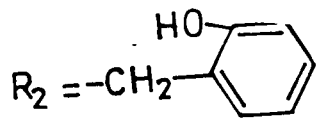
[9] R = H
[10] R = Me

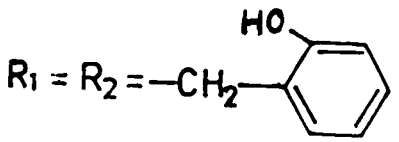


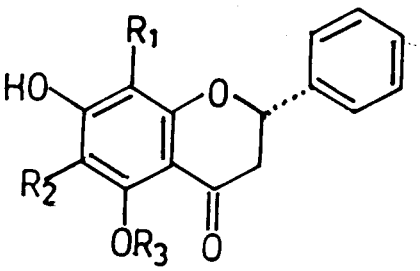
[11] R = H
[12] R = Me

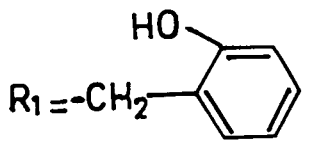


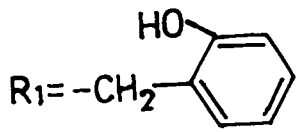
[16]  , R_2=H

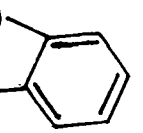
[17]  , R_1= H

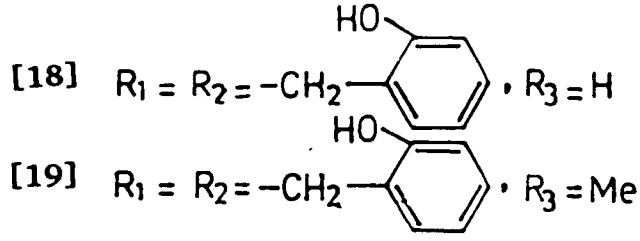
[20] 

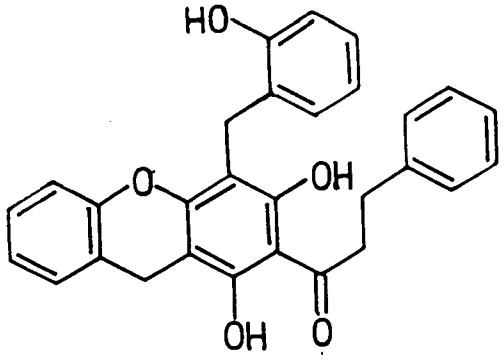


[13]  , $R_2 = R_3 = H$

[14]  , R_2 = H , R_3 = Me

[15] $R_1 = R_3 = H$, 





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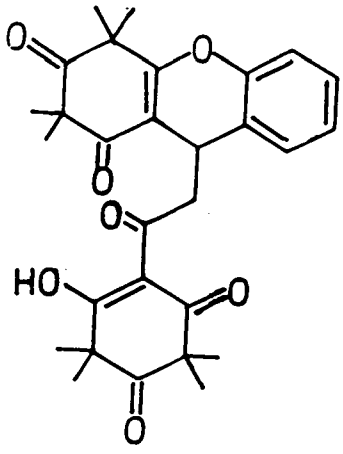

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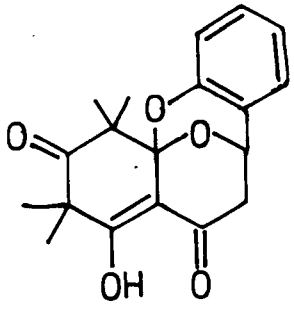

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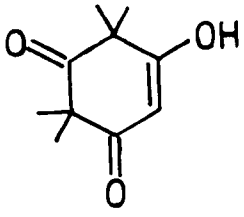

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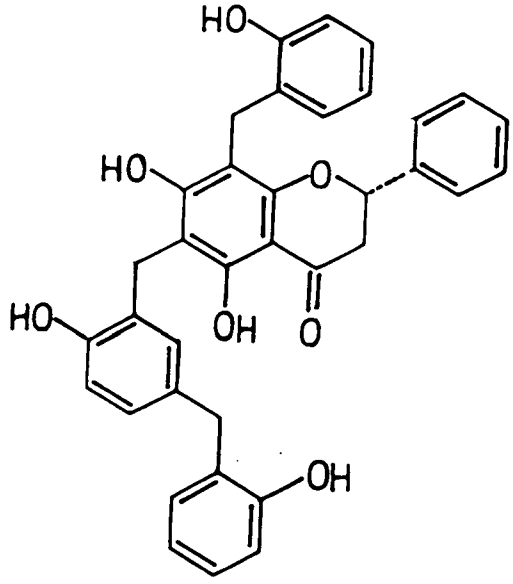
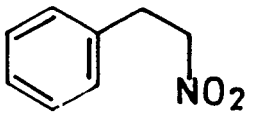


[22]

Essential oils

Many members of Annonaceae are fragrant due to the presence of essential oils. The constituents of these oils are usually either well known monoterpenes and sesquiterpenes or aromatic compounds. A large number of species are studied for their essential oil components. These include A. muricata, A. senegalensis, Xylopia longifolia, X. striata, X. aethiopica[151], X. brasiliensis etc. The most widely studied essential oil is ylang-ylang oil obtained from the flowers of Cananga odorata.[152] It is of considerable economic importance due to its wide spread use in perfumery. A very large number of constituents has been identified in this oil[134]. The major constituents of the seed oil of Annona squamosa are α-pinene, and caryophyllene while fruit peel oil contained mainly α -and β-pinene, limonene, β-farnesene and trans-ocimene. Leaf oil on the other hand contained α-pinene and caryophyllene and a sesquiterpene.[134] The essential oil of Xylopia aethiopica, which is used as a substitute for pepper in tropical Africa is found to contain β-pinene, cuminal, cineole, and terpinen-4-ol. Seeds of Dennetia tripetala is found to contain a rare compound, 3-phenylnitroethane [26].[153] Volatile oils from Polyalthia longifolia showed the presence

of azulenes and this oil is reported to have antimicrobial activity.



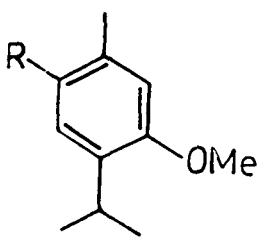
[26]

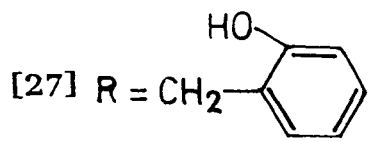
The essential oil from the root bark and leaves of U. chamae showed the presence of about fifty five constituents. The root oil contained a number of oxygenated benzyl benzoate derivatives and ethers. Thymoquinoldimethyl ether and benzyl benzoate are the major components of root oil.[154] The leaf oil contains predominantly sesquiterpene hydrocarbons, oxygenated sesquiterpenes, monoterpene hydrocarbons as well as oxygenated ones. These include T-cadinol, β-caryophyllene, caryophyllene oxide, β-elemene etc. Constituents like butyl acetate, T-cadinol and benzyl benzoate are detected in both leaf and root oil.[154] Uvaria narum root oil was found to show antimicrobial, anthelmintic, antifungal and central nervous system depressant activities and recent investigations lead to the isolation of about 52 compounds of which 25 are identified.[155] Uvaria narum root oil is found to be different from the root oil of other Uvaria species such as U. chamae and U. purpurea in that these species are rich in

aromatic compounds like benzyl benzoate[154,156,157], where as no such aromatic compounds are detected in the U. narum root oil.

Monoterpenes

A large number of monoterpenes are isolated from many members. U. chamae is found to contain a novel C-benzylated monoterpene chamanen [27] together with thymoquinol dimethyl ether [28] in its root bark.[146,157]. The presence of an o-hydroxybenzyl group in the molecule of chamanen recalls the special structure of the C-benzylated flavonoids of Uvaria.





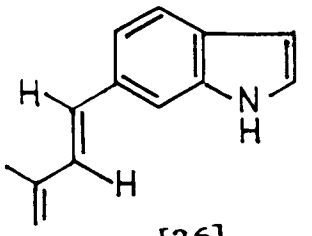
Sesquiterpenes

Numerous sesquiterpenes, some of them having biological activities, are reported from this family. Annona

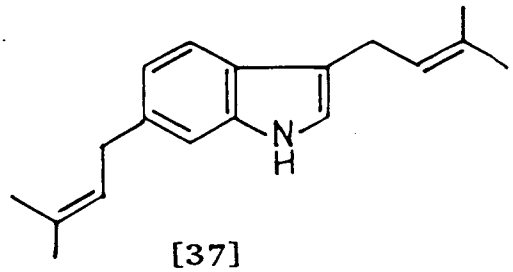
senegalensis for example, yielded a mixture of sequiterpenes possessing larvicidal properties.[134,158] In addition to the commonly occuring sesquiterpenes, certain genera like Uvaria, Polyalthia are found to elaborate sesquiterpenes of a special type containing an indole nucleus. The names, structures and sources of some of these compounds are given in the Table II. 2

Table II.2. Some of the nitrogenous sesquiterpenes isolated from the Annonaceae species

Name	Structure	Species	Reference
Polyalthenol	29	P. oliveri	159
Isopolyalthenol	30	P. suaveolens	134
Neopolyalthenol	31	P. suaveolens	134
Polyveoline	32	P. suaveolens	160,161
Polyavolensine	33	P. suaveolens	162
Polyavolensinol	34	P. suaveolens	162
Polyavolensinone	35	P. suaveolens	162
Isoprenylindole	36	Monodora tenufolia	160
3,6 Bis-dimethyl allylindole	37	U. elliotiana	163
3-Farnesylindole	38	U. pandensis	164
(6,7,-Dihydro-8,9 - dihydroxy) 3-farnesylindole	39	U. pandensis	165
(8,9, Dihydroxy) 3-farnesylindole	40	U. pandensis	165



[36]



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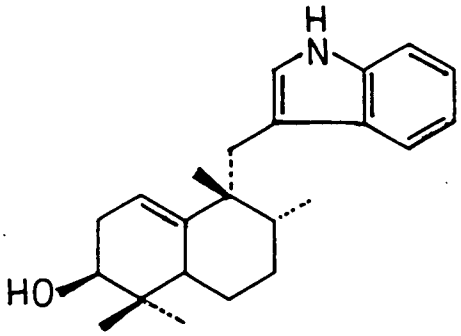
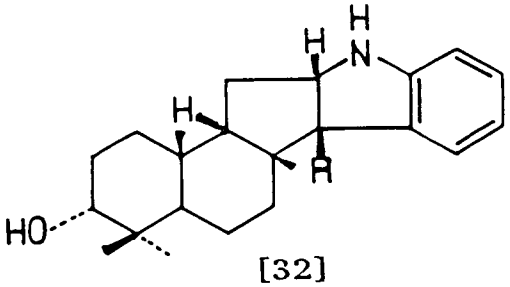
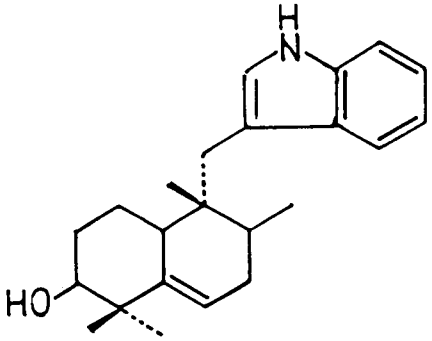


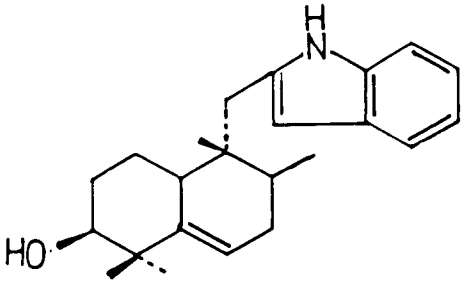
[29]



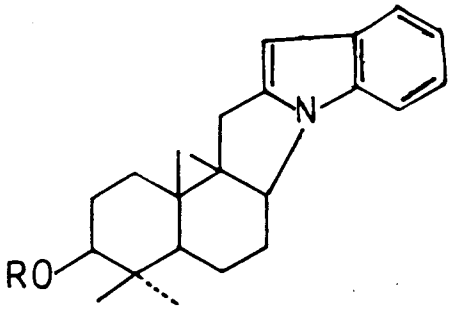
[32]



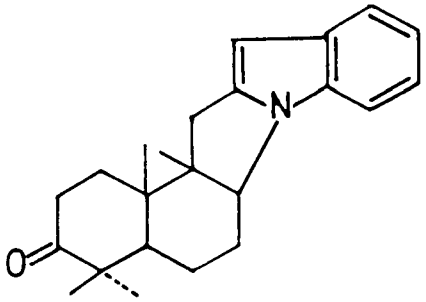
[30]



[31]



[33] R = Ac
[34] R = H



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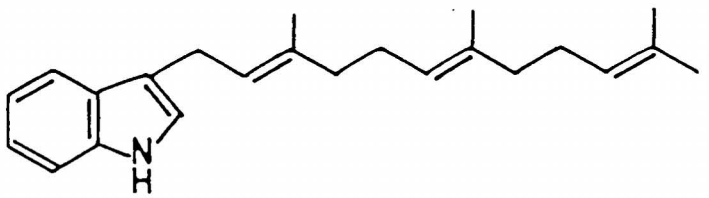

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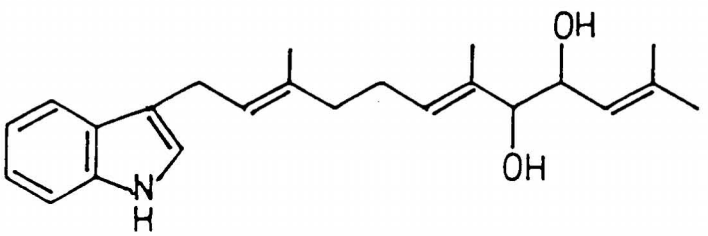

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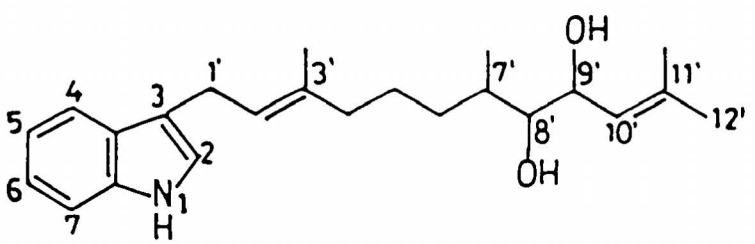
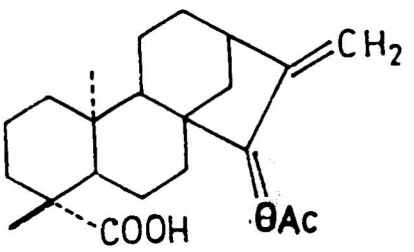


[39]

Diterpenes

About twenty kaurane type diterpenes have been reported from several Annona species.[167,168,169] One of this, Xylopic acid[41] is found to have antimicrobial activities.[166]

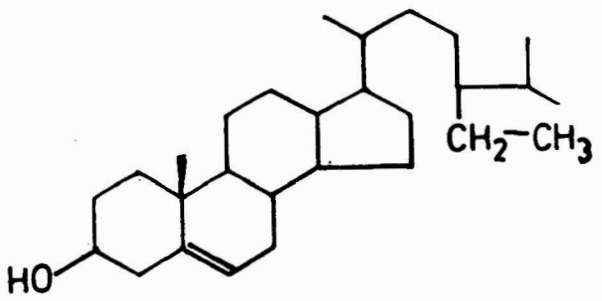


[41]

Triterpenes, Sterols and Saponins

Phytochemical screening tests have revealed the presence of many triterpenes and sterols in many Annonaceae members. β-Sitosterol [42] has been isolated from many members like A. muricata, A. squamosa and Polyalthia longifolia[170-173]. Sometimes, β-sitosterol is found to coexist with stigmasterol [43], campesterol [44] and cholesterol [45]. Other triterpenes isolated include friedelin [46], taraxerol [47], glutinol [48], glutinone

[49] and β-amyrin [50]. Taraxerol has been identified in U. scandens, U. narum[174], and U. scabrida. Glutinol is present in U. scheffleri[175] and in U. scabrida. Glutinol together with glutinone is present in U. sorzogonensis and U. narum[174]. A new tetracyclic triterpene polycarpol [51] has first been isolated from the bark of Polyalthia oliveri[159,176] but later found to be present in several other Annonaceae members. Polycarpol doesn't seem to have isolated from members of other family, this triterpene may thus be used as a chemotaxonomic marker.



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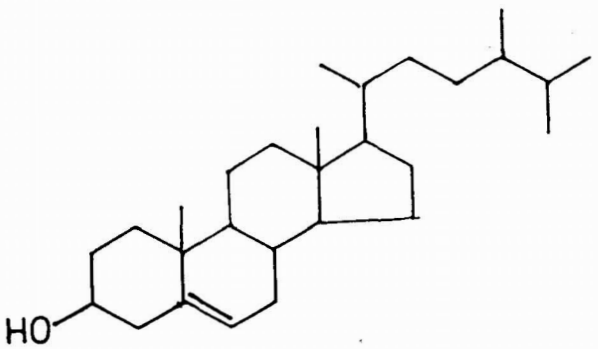
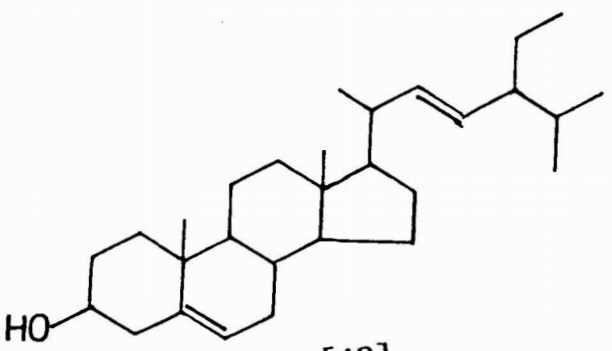
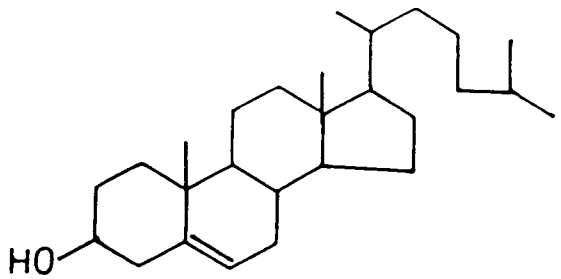


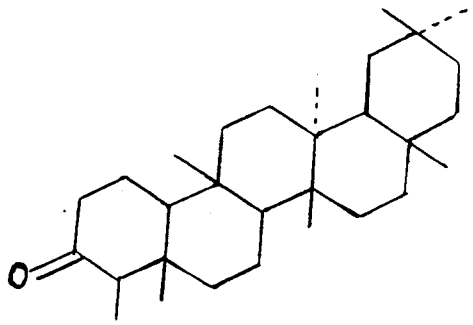
[44]



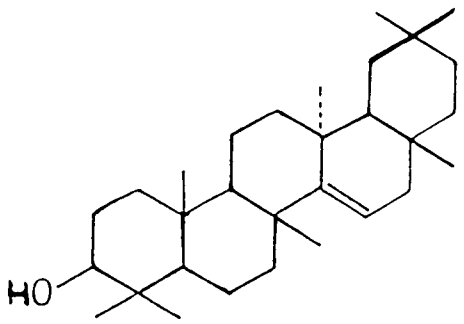
[43]



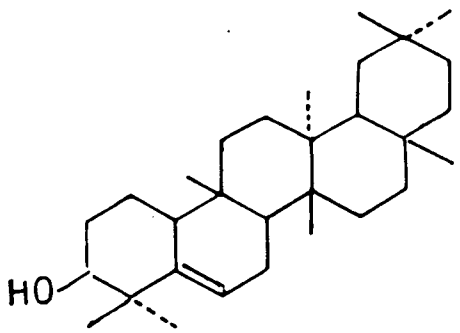
[45]



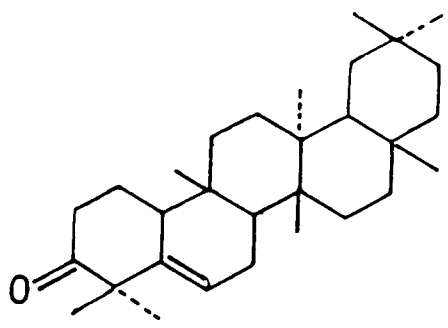
[46]



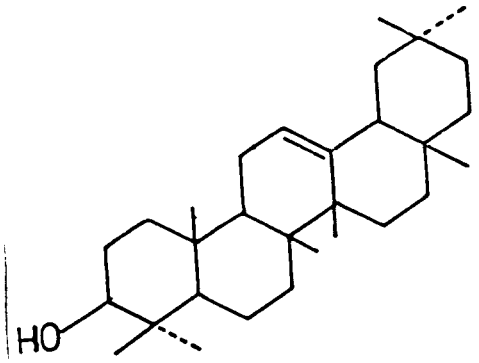
[47]



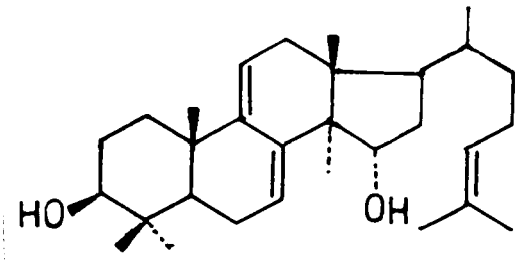
[48]



[49]



[50]



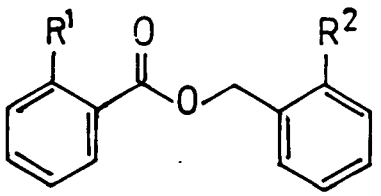
[51]

Aromatic Compounds

Certain genera of the Annonaceae, particularly Uvaria species are found to elaborate aromatic compounds. Benzyl benzoate or its derivatives are quite often present in many Annonaceae members. Benzyl benzoate [52], o-methoxybenzyl benzylether [53], o-methoxybenzyl benzoate [54] and di-o-methoxybenzyl ether [55] are isolated from the root bark of U. chamae, in addition to the isolation of a novel monobenzylated monoterpene chamanen.[157] Nine benzyl benzoates are isolated from the root of U. purpurea.[156] They are benzyl benzoate [52], 2-methoxybenzyl benzoate

[54], benzyl 2-hydroxybenzoate [56], benzyl 2-methoxybenzoate [57], benzyl 2,6-dihydroxybenzoate [58], benzyl 2-hydroxy-6-methoxybenzoate [59], benzyl 2,6-dimethoxybenzoate [60] benzyl 2-hydroxy,5-methoxybenzoate [61] and benzyl 2,5-dimethoxybenzoate [62]. Benzyl 2,3,6-trimethoxybenzoate was obtained from the stem bark of U. ovata [63].

From studies on species like Uvaria ferruginia[177], Uvaria ovata[178] and Uvaria kirki[147], it appears that a general theme running through the chemistry of Uvaria is the ability to employ benzyl or benzoyl groups to substitute to a number of different kinds of secondary metabolites.

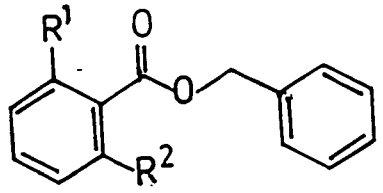


[52] $R^1 = R^2 = H$

[54] $R^1 = H$, $R^2 = OMe$

[56] $R^1 = OH$, $R^2 = H$

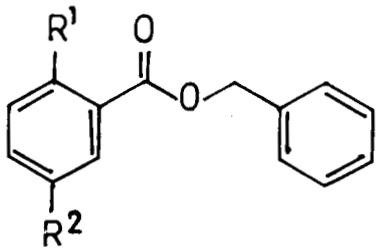
[57] $R^1 = OMe$, $R^2 = H$



[58] $R^1 = R^2 = OH$

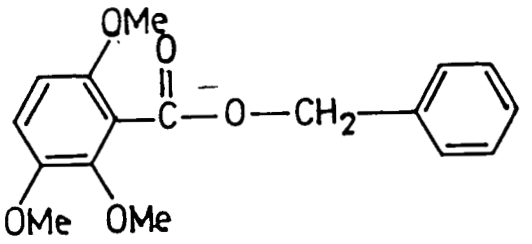
[59] $R^1 = OH$, $R^2 = OMe$

[60] $R^1 = R^2 = OMe$

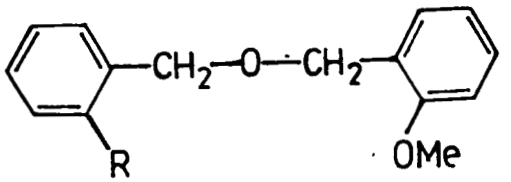


[61] $R^1 = OH$, $R^2 = OMe$

[62] $R^1 = R^2 = OMe$



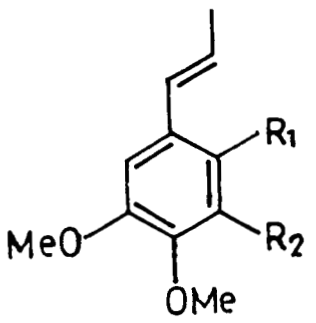
[63]



[53] R = H

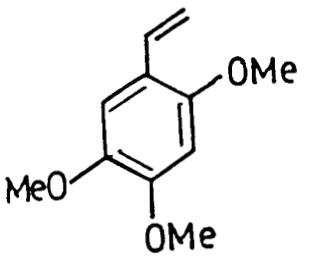
[55] R = OMe

Several propenyl and vinylbenzene derivatives have been recorded from Annonaceous plants. Asarone [64] and trans-isoelemicin [65] are isolated from the bark of Guatteria gaumeri[179] where as 2,4,5-trimethoxystyrene [66] is isolated from Pachypodanthium staudtii.[180]



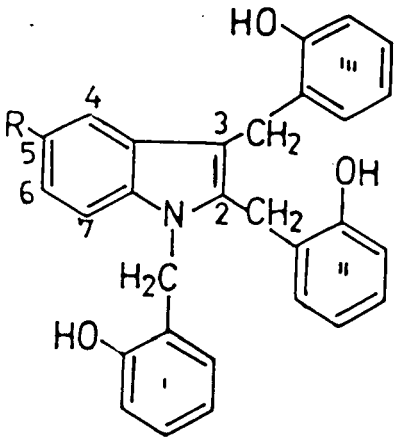
[64] $R_1 = OMe$, $R_2 = H$

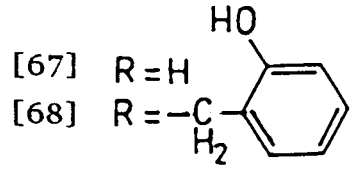
[65] $R_1 = H$, $R_2 = OMe$



[66]

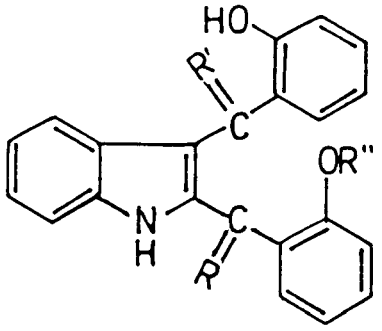
A recent study on Uvaria angolensis revealed the presence of three novel benzylated indoles namely Uvarindole A,B,C [67,68,69] and three benzylated sesquiterpenes namely Uvarisesquiterpens A,B and C [70,71,72]. U. scheffleri contains another kind of new metabolites called panduratins which are considered to be biogenetically derived from a Diels Alder like cyclisation between a chalcone and a monoterpene, β-ocimene.[181]





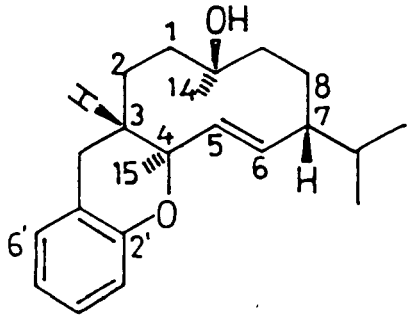
[67] R = H

[68] R = $-CH_2-$

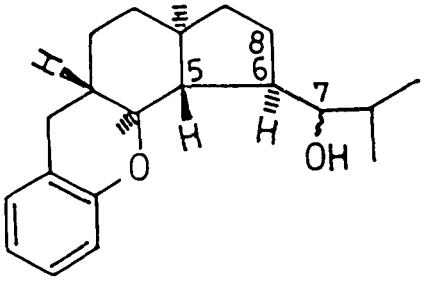


R = O R' = H_2 R'' = H

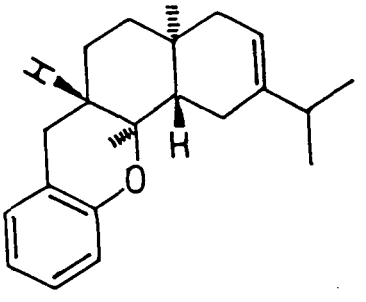
[69]



[70]



[72]



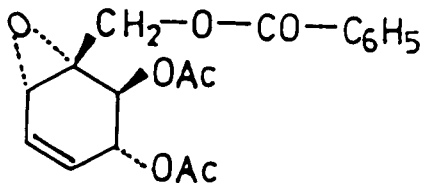
[71]

Cyclohexene epoxides

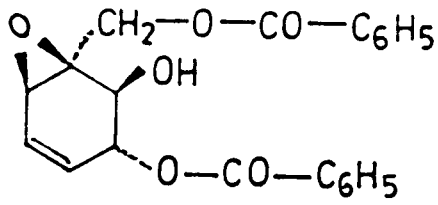
Investigations on several species of Uvaria lead to the isolation of another class of secondary metabolites which are poly-oxygenated cyclohexene derivatives incorporated with benzoyl esters. These compounds are not aromatic, because the aromatic ring has been further oxidized to an epoxide. These compounds display an interesting spectrum of biological activities. They form a small class of plant secondary metabolites and interest in this unusal compounds has raised the question of their biogenesis. The names and sources of some of these compounds are listed in Table II.3.

Table II.3 Cyclohexene epoxides isolated from Uvaria species

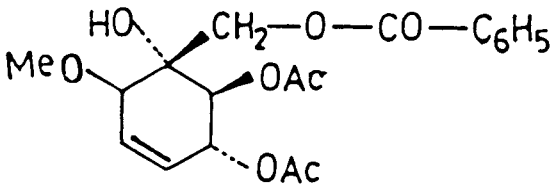
Name	Structure	Species	References
Senepoxide	73	U. catocarpa	166
Seneol	74	U. catocarpa	167
Pipoxide	75	U. purpurea	182
		U. pandensis	183
1,6-Deoxy senepoxide	76	U. ferruginia	184
1,6 - Deoxy tinganoxide	77	U. ferruginia	184,177
α-Senepoxide	78	U. ferruginia	184,177
α-Tinganoxide	79	U. ferruginia	"
β-Senepoxide	80	U. pandensis	183
Pandoxide	81	U. pandensis	183
Uvarigranol A	82	U. grandiflora	185,186
Uvarigranol B	83	"	"
Zeylenol	84	"	186
Uvarigranol C	85	"	187
Uvarigranol D	86	"	187



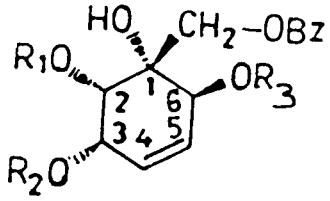
[73]



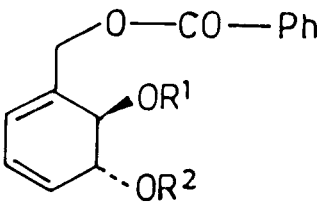
[75]



[74]

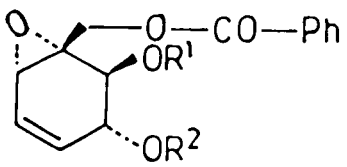


	R_1	R_2	R_3
[84]	H	Bz	H
[82]	H	H	Bz
[83]	Ac	Bz	H
[85]	H	Bz	Et
[86]	Ac	Bz	Et



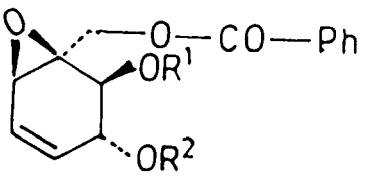
[76] $R^1 = R^2 = COMe$

[77] $R^1 = COMe$, $R^2 = COPh$

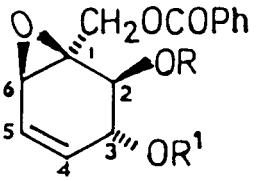


[78] $R^1 = R^2 = COMe$

[79] $R^1 = COMe$, $R^2 = COPh$



[80] $R^1 = R^2 = COMe$



R = COMe, R^1 = Me

Alkaloids of Annonaceae

The literature on the alkaloidal components from Annonaceae is abundant. In general, almost all alkaloids of Annonaceae possess an isoquinoline derived structure and they can be subclassed as simple isoquinolines, benzyl tetrahydroisoquinolines, bisbenzylisoquinolines, bisbenzyl tetrahydroisoquinolines, protoberberins, tetrahydroprotoberberins, aporphinoids, 7-substituted aporphines, oxoaporphines, phenanthrenes and other miscellaneous isoquinolines. Recently investigations on the leaves of Annona montana lead to the isolation of a novel cytotoxic phenanthrene alkaloid together with two known cytotoxic alkaloids and an oxoaporphine alkaloid which is not bioactive.[188]

Acetogenins of Annonaceae

Recent investigations on some species in the genera such as Uvaria, Rollinia, Annona and Goniothalamus have resulted in the isolation and characterization of several novel acetogenins, which are now referred to as Annonaceous acetogenins. These are a series of polyketide derived fatty acid derivatives. They possess one or two tetrahydrofuran (THF) rings and a methylated γ-lactone unit (usually α-β-

unsaturated and sometimes rearranged to a methyl keto lactone), with various hydroxyl, acetoxyl and or ketoxyl groups along the hydrocarbon chain. These compounds exhibit a wide range of bioactivities such as cytotoxicity, antitumor, anthelmintic, antimalarial, antimicrobial, immunosuppressant, antifeedant, pesticidal and other miscellaneous activities.

These compounds have attracted much interest not only because of their bioactivities, but also because of their interesting structural features.

About one hundred and twenty eight different linear, epoxy, monotetrahydrfuran, (mono-THF) bistetrahydrofuran (bis-THF) and tritetrahydrofuran (tri-THF) acetogenins have been isolated from Annonaceae. Application of combined techniques has been necessary to facilitate the structural elucidation of the different parts of these complex molecules[189-193]. Uvaricin was the first member in this extremely bioactive compounds, which was reported in 1984, as a potential antitumor agent from U. accuminata by Jolad and coworkers.[185] To date four linear acetogenins,[194-197] 12 epoxy[198-199] 51 mono-THF,[191,200-203] 60 bis-THF[204-223] and one tri-THF acetogenin have been isolated from this family.

The extraction and isolation procedure of acetogenines differ in many cases depending on the nature of plant material and the plant part under study. However, ethyl acetate extraction, after hexane extraction is an efficient extraction procedure. Chromatographic techniques are used to isolate pure compounds.

A good number of acetogenins have already tested for their cytotoxicities and antitumor activities. Testing of various mono-THF, nonadjacent bis-THF and adjacent bis-THF acetogenins for their antitumour, and cytotoxic effects illustrate their structure-activity relationship. It has been shown that the most potent cytotoxic compounds possess an adjacent bis-THF subunit. Nonadjacent bis-THF acetogenins show lesser cytotoxicity and the mono-THF acetogenins are the least cytotoxic. Hydroxyl groups are also found to play an important role in cytotoxicity because, the acetylation of hydroxyl groups leads to decrease in cytotoxicity and the reduction of keto group increases cytotoxicity. Recent biochemical work has reported that the mode of action of acetogenins targets on the mitochondrial NADH: Ubiquinone oxidoreductase, also known as the respiratory complex I of mitochondria. The ability of the bioactive Annonaceous acetogenins to inhibit mitochondrial respiration at site I was first noted by

Londenshausen and coworkers[224] and confirmed by several authors.[225,226] Extensive studies on the mechanism by which the Annonaceous acetogenins inhibit the complex I are currently in progress.[227]

Many of the acetogenins are found to show pesticidal activities. In 1988, Mikolajczak and coworkers patented Annonaceous acetogenins as pesticides.[228] The acetogenins annonin and neoannonin showed strong ovicidal activities. The crude extract of Asimina triloba (which contains asimicin) shows promise as a garden pesticide. Many acetogenins showed selected pesticidal activities against certain pests. A mixture of these compounds can show useful additive properties and synergistic effects which would extend the spectrum of pests affected. These extracts can have application as biodegradable pesticides. Studies show that the terminal lactone could be responsible for the pesticidal activities, since ketolactone structures didn't show any activity at the same dose level.

The acetogenins isolated from Annona squamosa shows anthelmintic effects against Caenonhabditis elegans.[229,230] This explains the use of Annona squamosa in folk medicine in treatment of worm diseases. Several acetogenins are also used as antimitotic agents. Some of the acetogenins like

asimicin, annonacin, goniothalamicin etc. are found to show antimalarial activity, equal or superior to tetracycline. The α-β-unsaturated compounds are found to be much more active than the reduction products. The stereochemistry of bis-THF part also plays an important role in its activity.

The interesting bioactivities of these compounds provoke workers in this field to explore further on their structure-activity relationship. Obviously much work remain to be done to develop successful applications for the naturally occuring Annonaceous acetogenins and their derivatives in the biochemical, medical and agro chemical fields. The isolation and characterization of Annonaceous acetogenins validate the folkloric claims and uses of some of these plants in traditional medicine.

Miscellaneous compounds

In addition to the compounds mentioned above, several other classes of compounds like polyacetylenes, carotenes, vitamines, cyanogenic glycosides, nonalkaloidal nitrogen heterocycles etc. have been isolated from many members.

Conclusion

The above survey indicates that the literature on

Annonaceae has grown considerably in the last decade and now a vast field is open to chemists and pharmacologists for in-depth investigations. Of more than 2000 species not more than 15% have been investigated so far. Moreover, many earlier studies are only fragmentary and systematic reinvestigations are necessary in many cases. In order to draw valid conclusions on chemotaxonomic features of the Annonaceae, still more chemical investigations are necessary.

More over some of the alkaloidal and non alkaloidal constituents are found to be pharmacologically important either individually or they belong to a group which is known to produce many medicinal compounds. All the studies so far conducted on the Annonaceae plants prove the possibility for chemists and pharmacologists to go further to explore more new compounds with interesting bio-activities.

SECTION 2: PHYTOCHEMICAL STUDIES ON THE LEAVES OF UVARIA NARUM

Introduction

Uvaria narum (Annonaceae) commonly called 'pandel' in Tulu and 'Narum panel' in Malayalam is a shrub or sometimes a woody climber growing in the western ghats of India, as well as in the plaines of Kerala. According to literature[231,232] this plant is used in folk medicine for various purposes. This plant is said to have insecticidal properties. The leaves are recommended in rheumatic swellings, jaundice, biliousness and typhoid. A decoction of the root bark is given to women to control fits at the time of delivary. It is also used in rheumatism, bowels complaints of children and for eczema.

II.1. The aim and scope of present investigation

The benzene, ethyl acetate and aqueous extracts of Uvaria narum leaves showed pronounced antifungal activity against the fungus, 'Phytophthora capsici' which causes the devastating disease 'foot rot', of pepper. TLC Analysis of the petroleum ether, benzene and ethyl acetate extracts showed the presence of many compounds. A systematic

phytochemical investigation was therefore carried out with a view to isolate the antifungal principles and to characterize as many constituents as possible from these extracts.

II.2. Phytochemical and Pharmacological studies so far reported on Uvaria narum

A literature survey on Uvaria narum showed the following works reported on this plant.

Pharmacological evaluation of its root bark oil is reported in 1988 with the identification of a few volatile compounds.[233] In this study, Uvaria narum root bark oil is reported to show very good antimicrobial activity against Bacillus megathecium and Escherichia coli, pronounced antifungal activity against Candida albicans, but no activity on Aspergillus niger. It also shows strong anthelmintic activity compared to piperazine phosphate. The oil significantly potentiated sodium pentabarbitone induced hypnosis in mice. The oil is found to be a good potentiator, but doesn't have much sedative action of its own. It is a central nervous system depressant and this effect suggests its usefulness as a tranquilizer. Qualitative study of the oil by GC-MS revealed the presence

of five compounds, β-caryophyllene and bornyl acetate being the major constituents. The oil is also reported to contain several unidentified sesquiterpenes.

Hisham and coworkers[155] identified 25 compounds from the root essential oil by GC-MS analysis. Monoterpenes contributed to about 23.7% of the total oil. Among this bornyl acetate, borneol and an unidentified monoterpene alcohol are the major constituents. Sesquiterpene hydrocarbons contributed to 41.3% of the total oil of which 18 are identified. Oxygenated sesquiterpenes contributed to 29.2% of the total oil. Among this only one compound, patchoulenone, an uncommon tricyclic sesquiterpene ketone could be identified. It is also reported that the root oil of other species such as Uvaria chamae, Uvaria purpurea[153,156, 157] contain large amounts of aromatic compounds such as benzyl benzoate and its derivatives. In the case of root oil of U. narum, no such aromatic compound is detected. Phytochemical studies on the root bark lead to the isolation of compounds such as glutinol, glutinone, taraxerol, β-sitosterol, benzyl benzoate and patchoulenone.[174] In addition, several annonaceous acetogenins have been isolated from the root bark of Uvaria narum.[155,174]

The hexane and ethyl acetate extract of root bark of U. narum showed high inhibition against the fungi, Candida, Penicillium and Aspergillus; good inhibition against a range of bacterial species and anthelmintic activity comparable with standard drugs like mebendazole. These activities are attributed to the acetogenins isolated from these extracts.[234].

Recent investigations[235] on the leaves of U. narum lead to the isolation of four compounds, tritriacontane, tetratriacontanol, β-sitosterol and a new benzoic acid ester, 2-E(2"-oxo-cyclopent-3"-en-1"-ylidene)ethyl benzoate.

II.3. MATERIALS AND METHODS

The plant material used in this investigation was collected during December-January from the Calicut University Campus, Malappuram district of Kerala and was identified by Dr. A.K. Pradeep, Dept. of Botany, Calicut University.

Experimental set up for TLC, column chromatography, IR spectra and mass spectra were the same as that described in chapter I. ^{1}H NMR spectra were recorded using Bruker spectrometer at 500 MHz and 250 MHz in $CDCl_3$. ^{13}C NMR Spectra were recorded using Bruker spectrometer at 63 MHz

and 125 MHz. High resolution mass spectra were recorded using Finnigan MAT 95. All the melting points of the crystalline isolates were determined using Toshniwal Capillary Melting Point Apparatus and are uncorrected.

II.4. Extraction, fractionation and isolation of compounds from the leaves of Uvaria narum

II.4.1. Fractionation of the petroleum ether extract of the leaves of Uvaria narum

Dried and finely powdered leaves (2 Kg) of Uvaria narum were extracted thrice with petroleum ether [3 x 6L; 60-80^{o}C]. The combined petroleum ether extract was concentrated to get about 8 g of viscous oily material which was subjected to column chromatography (5 cm x 100 cm; dxl) over silicagel (70-120 mesh). The column was developed using petroleum ether and eluted with solvents of increasing polarity (Petroleum ether, benzene, benzene-ethyl acetate, ethyl acetate). Several 50 ml portions were collected and every fraction checked by TLC. Identical portions were pooled together. Various fractions, eluent composition and components isolated from the petroleum ether extract on column chromatography are given in table II.4.1.

Table II.4.1. Chromatographic fractionation of the petroleum ether extract of Uvaria narum

Fraction	Eluent composition	Components
1	Petroleum ether	U_1
2	Benzene	U_2
3	4:1 Benzene-ethyl acetate	U_3
4	3:1 Benzene-ethyl acetate	U_4

II.4.2. Isolation of compounds from different chromatographic fractions of petroleum ether extract

Fraction 1, obtained on elution with petroleum ether, on evaporation gave a white crystalline solid which on recrystallisation from ethyl acetate gave 3.5 g of compound U_1, m.p. 55°C.

Fraction 2, deep red in colour, obtained on elution with benzene on evaporation gave red waxy solid. Column chromatography (3 cm x 100 cm; dxl) over silicagel (100-200 mesh) of this solid using petroleum spirit, gave light yellow powdery substance. This on repeated recrystallisation from acetone gave 100 mg of white powdery substance, U_2, m.p. 70°C.

Fraction 3, eluted out with 4:1 benzene-ethyl acetate was green in colour. This on evaporation gave a greenish solid. It was subjected to column chromatography (3cm x 100cm; dxl) over silicagel (70-120 mesh). Benzene was used to develop the column and eluted with benzene and 4:1 benzene-ethyl acetate mixture. The 4:1 benzene-ethyl acetate portion on evaporation gave a white powdery substance, which on recrystallisation from ethyl acetate yielded 200 mg of pure substance, U_3, m.p. 86°C.

Fraction 4, deep green in colour, on evaporation gave a green mass. TLC Examination of it gave a deep pink spot on spraying with Liebermann-Burchard reagent. It was dissolved in ethyl acetate and adsorbed on silica gel (150 g; 100-200 mesh). After drying it was taken in a chromatographic column and eluted with benzene followed by benzene-ethyl acetate mixture (4:1). The latter portion on evaporation and recrystallisation from benzene gave shining crystals of U_4 (400 mg, m.p. 137°C).

II.4.3. Fractionation of the alcohol extract of the leaves of Uvaria narum

Finely powdered leaves of Uvaria narum, after extraction with petroleum ether was extracted thrice with

alcohol (3 x 2 L). The combined alcohol extract was concentrated under reduced pressure to about 250 mL. Added about 250 mL water and extracted with benzene and ethyl acetate. Both the extracts were concentrated and chromatographed (3 x 100 cm ; dxl) over silicagel (100-200 mesh). For the benzene extract, the column was developed with benzene and eluted with benzene (1 L), 3:1 benzene-ethyl acetate (1 L), 2:1 benzene-ethyl acetate (500 mL) and ethyl acetate (500 mL). The 3:1 benzene-ethyl acetate effluent on evaporation gave a white crystalline compound U_5, recrystallised from benzene as long shining needles (1.5 g), m.p. 119°C.

Ethyl acetate fraction after evaporation was also subjected to column chromatography using silicagel (2 cm x 50 cm; dxl). 3:1 Benzene-ethyl acetate was used to develop the column and eluted with 3:1 benzene-ethyl acetate (250 mL) and ethyl acetate (250 mL). Ethyl acetate fraction gave a yellow shining crystalline solid, U_6 (2 mg), m.p. 255°C.

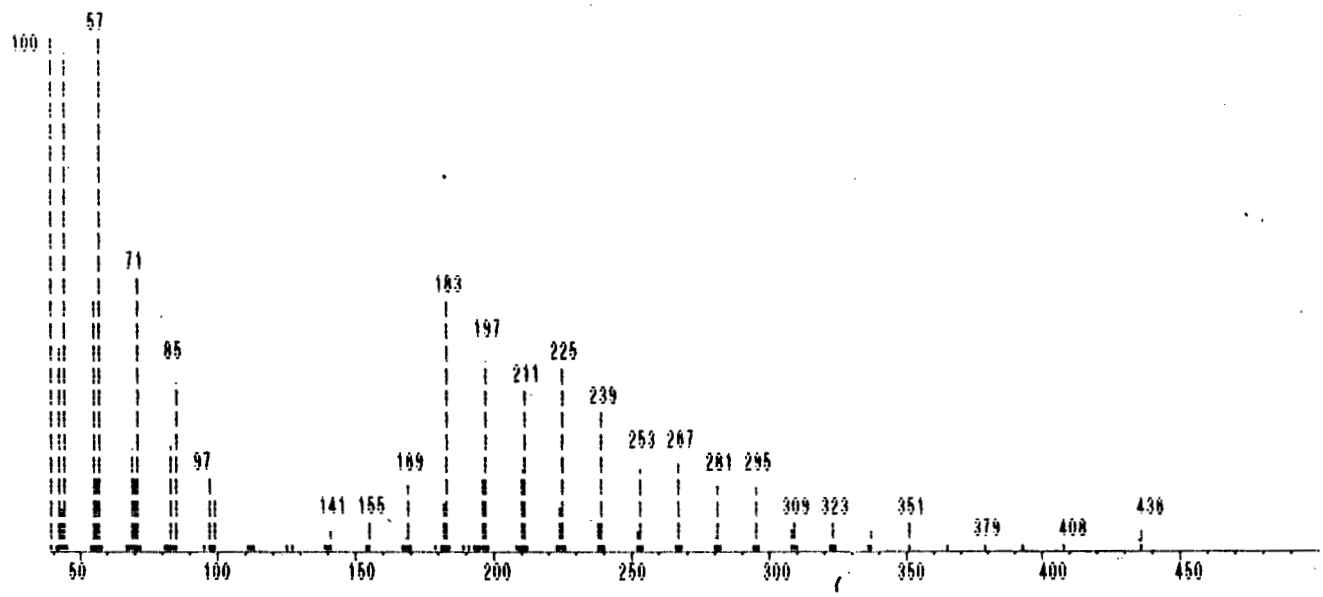
II.5. RESULTS AND DISCUSSION

II.5.1. Characterization of U_1

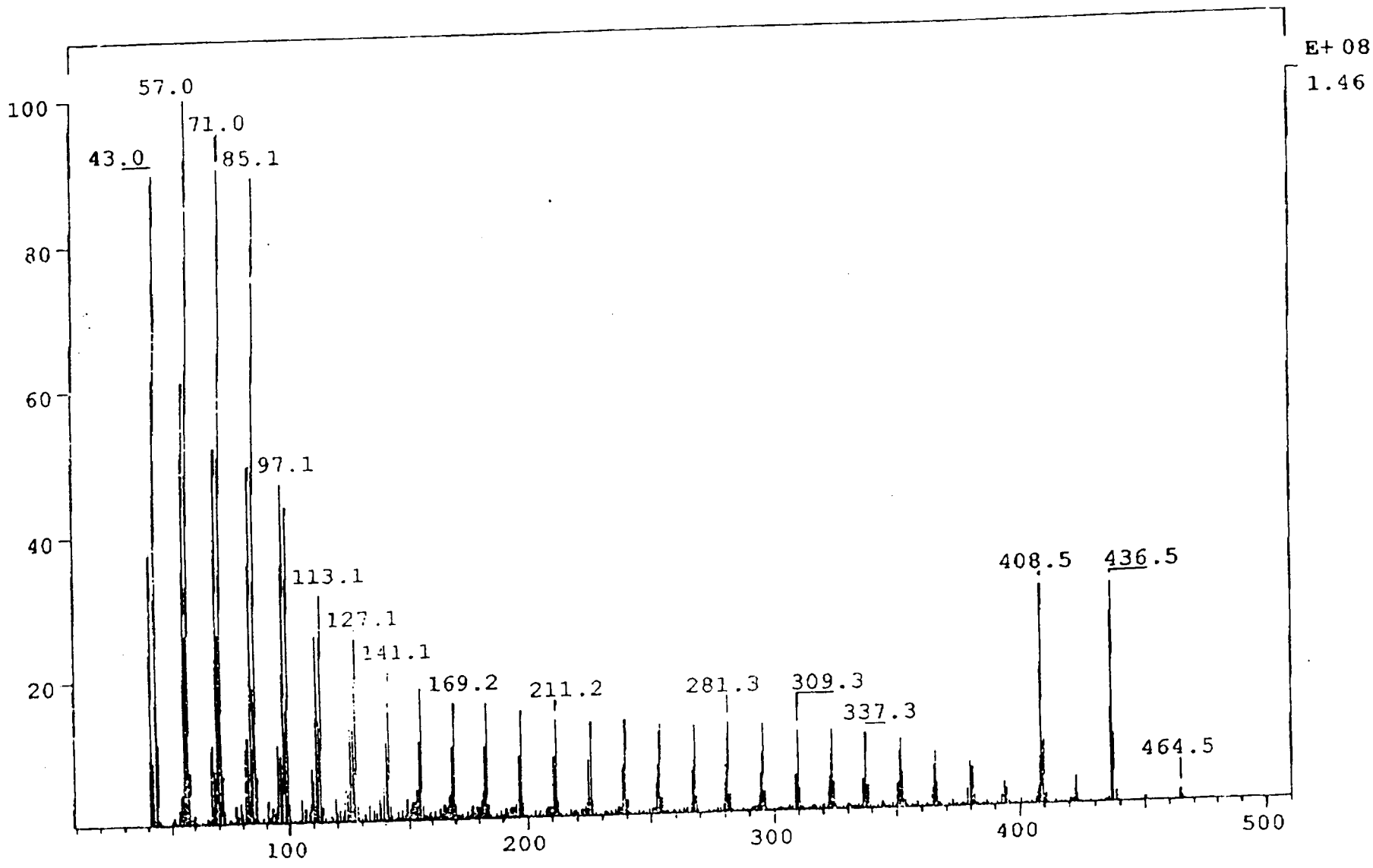
The compound U_1 isolated as described in section II.4.2. was a white shining crystalline solid having a m.p.

55°C. This compound didn't answer Liebermann-Burchard colour reaction, indicating that it was not a triterpenoid or a sterol. It gave a black colour on spraying with H_2SO_4 and strong heating. It moved as a single spot on TLC in petroleum ether and cyclohexane. Absence of decolourisation with dilute $KMnO_4$ solution and bromine water showed its saturated nature. EI Mass spectrum of this compound showed M^+ at m/z 436 and base beak at m/z 57. Fragmentation pattern with a regular difference of 14 mass units showed its straight chain saturated hydrocarbon nature. IR Spectrum showed absorptions due to -C-H stretching and -C-H bending (2957.2, 2916.7 cm^{-1} and 1473.8, 1464.1 cm^{-1} respectively). A long chain hydrocarbon nature was indicated by the absorptions at 729.2 and 719.5 cm^{-1}. ^1H NMR (absorptions in the range δ 0.86-1.32) and ^{13}C NMR (absorptions at δ 14.13, 22.7, 29.37, 29.70 and 31.9) confirmed the straight chain nature. Attached proton test (APT) also confirmed the straight chain nature of U_1 as it showed only-CH_2-and -CH_3 groups (^{13}C absorption of -CH_3 in -ve mode and -CH_2-absorptions in +ve mode). The M^+ ion at m/z 436 indicated the compound to be hentriacontane. But the reported m.p. of this compound is 68°C.[236] The low m.p. of U_1 (55°C) indicated it to be a mixture. Significant m/z values on high resolution mass spectrum were found at

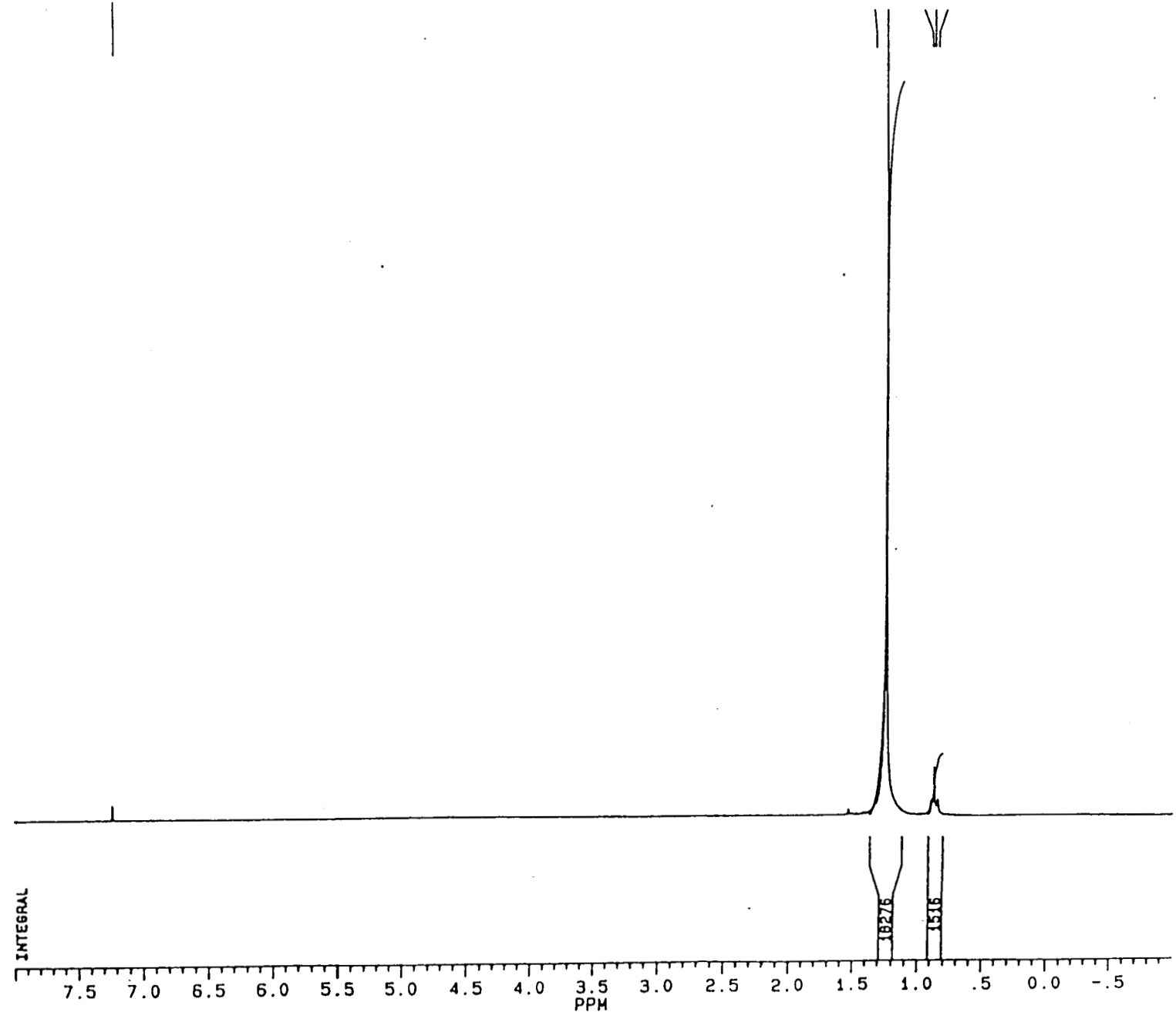
408.4695, 436.5008, 464.5321 corresponding to $C_{29}H_{60}$ (nonacosane), $C_{31}H_{64}$ (hentriacontane) and $C_{33}H_{68}$ (tritriacontane) respectively. U_1 was thus identified to be a mixture of nonacosane, hentriacontane, and tritriacontane.



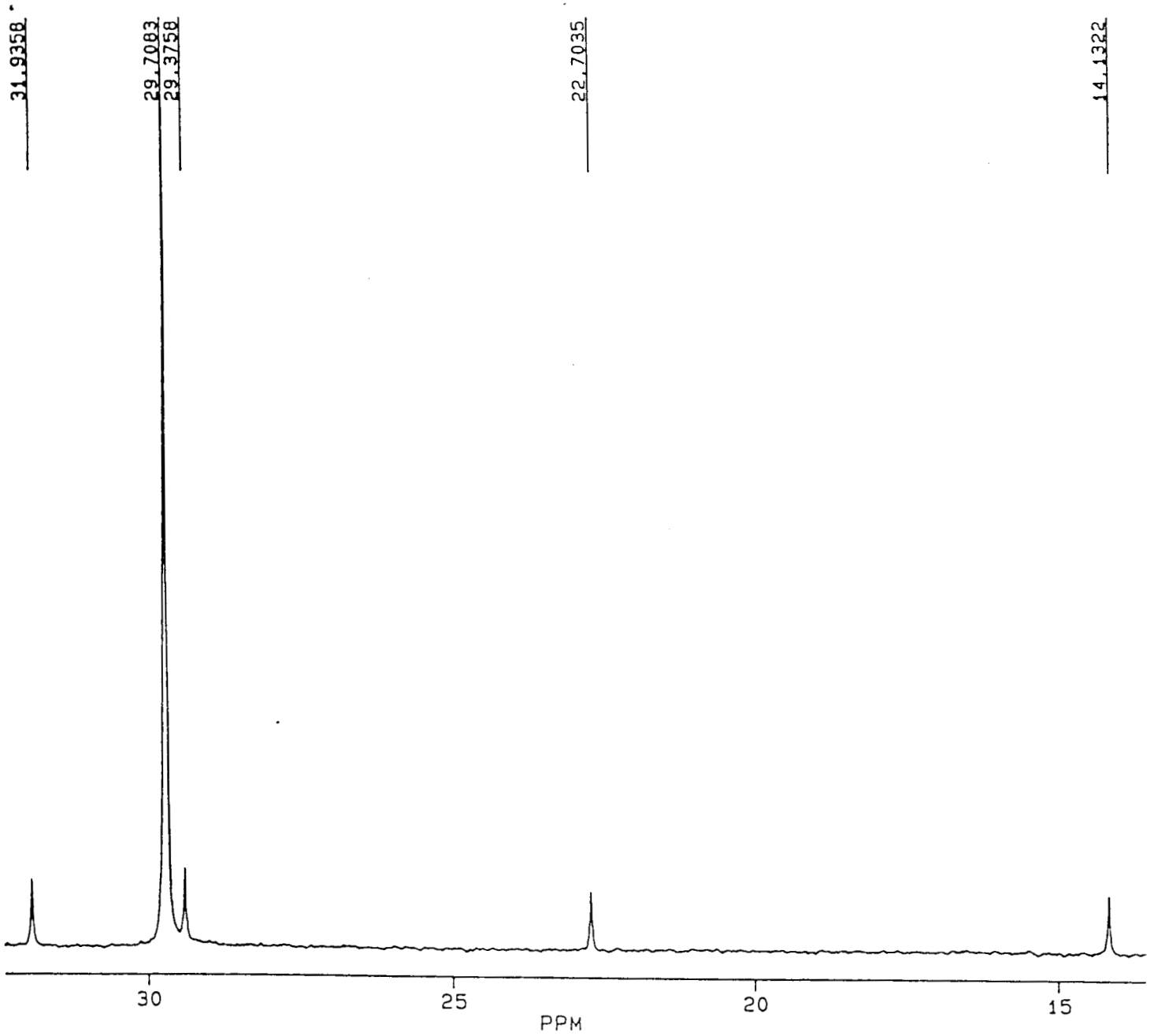
EI-mass spectrum of U_1 [Mixture of alkanes]



High resolution mass spectrum of U_1 [Mixture of alkanes]



^{1}H-NMR spectrum of U_1 [Mixture of alkanes] (250 MHz, $CDCl_3$, TMS)



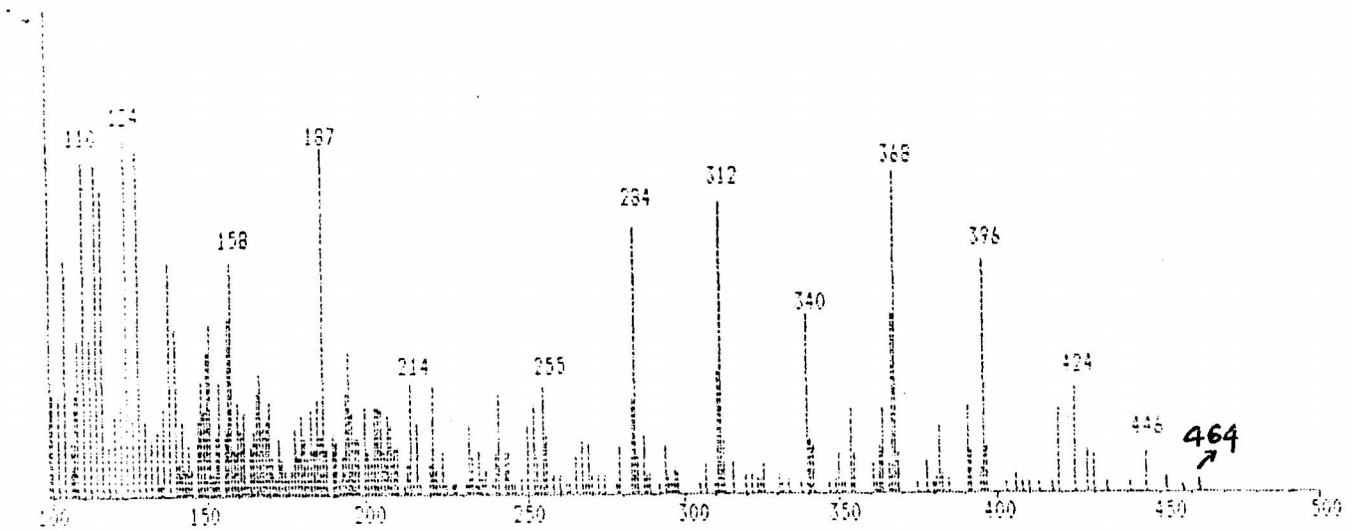
^{13}C-NMR spectrum of U_1 [Mixture of alkanes] (63 MHz, $CDCl_3$, TMS)

PPM
77.4991
76.9913
76.4831
31.9231
29.6953
29.3626
22.6903
14.1184
75
70
65
60
55
50
45
PPM
40
35
30
25
20
15

AM 13C 04 12

II.5.2. Characterization of U_2

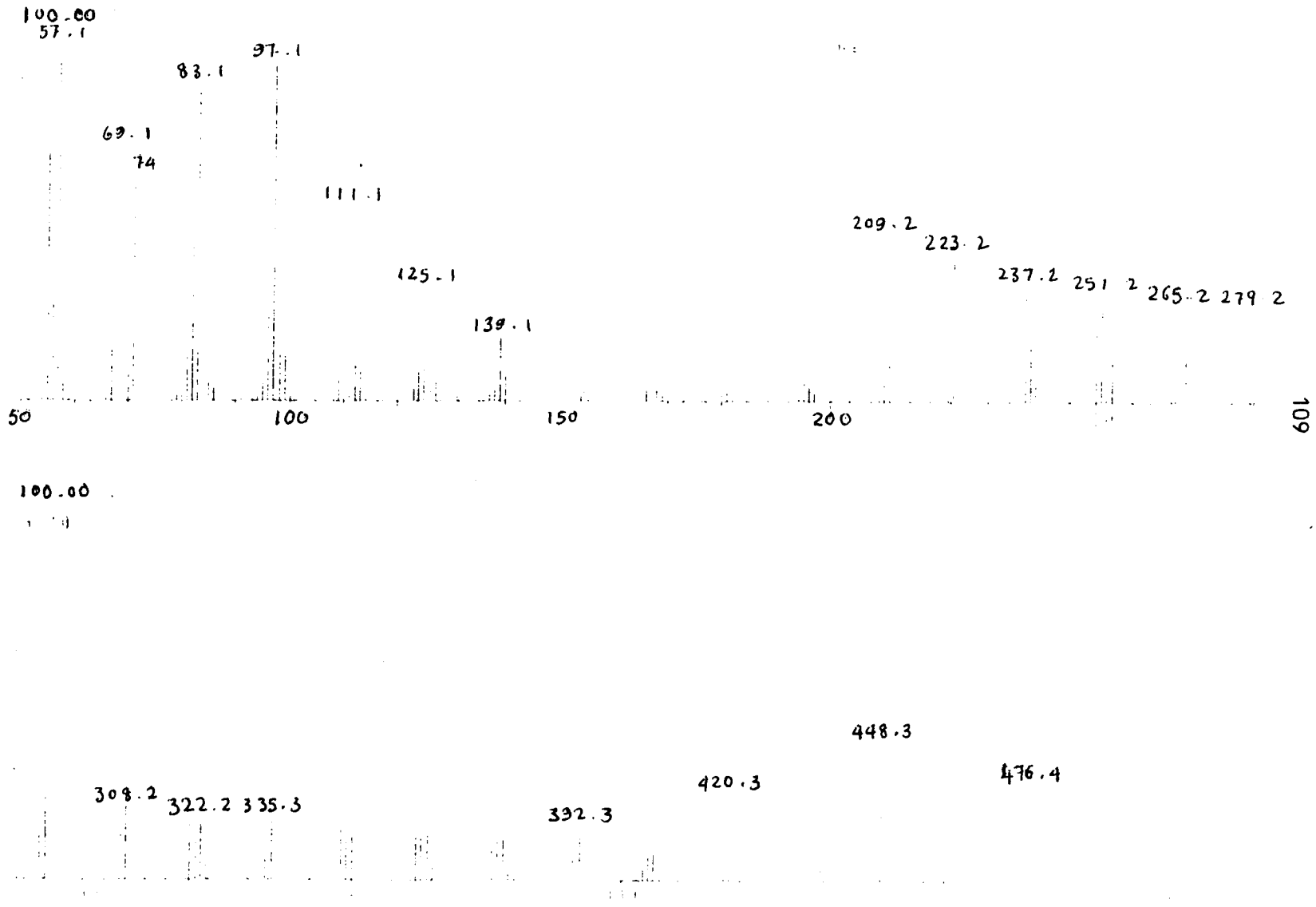
The compound U_2 was obtained from the petroleum ether extract on elution with benzene as described in section II.4.2. It also gave negative test with Liebermann-Burchard reagent. Absence of decolourisation with $KMnO_4$ solution and bromine water showed its saturated nature. It gave a brown spot on spraying with H_2SO_4 and strong heating. EI Mass spectrum of U_2 had M^+ at m/z 464 and base peak at m/z 57. Fragmentation pattern with a regular difference of 14 mass units showed its straight chain saturated hydrocarbon nature. IR Spectrum also confirmed this. Comparison of m.p. (70^oC) with that of literature (71.8^oC)[235,237] confirmed the identity of the compound as tritriacontane.



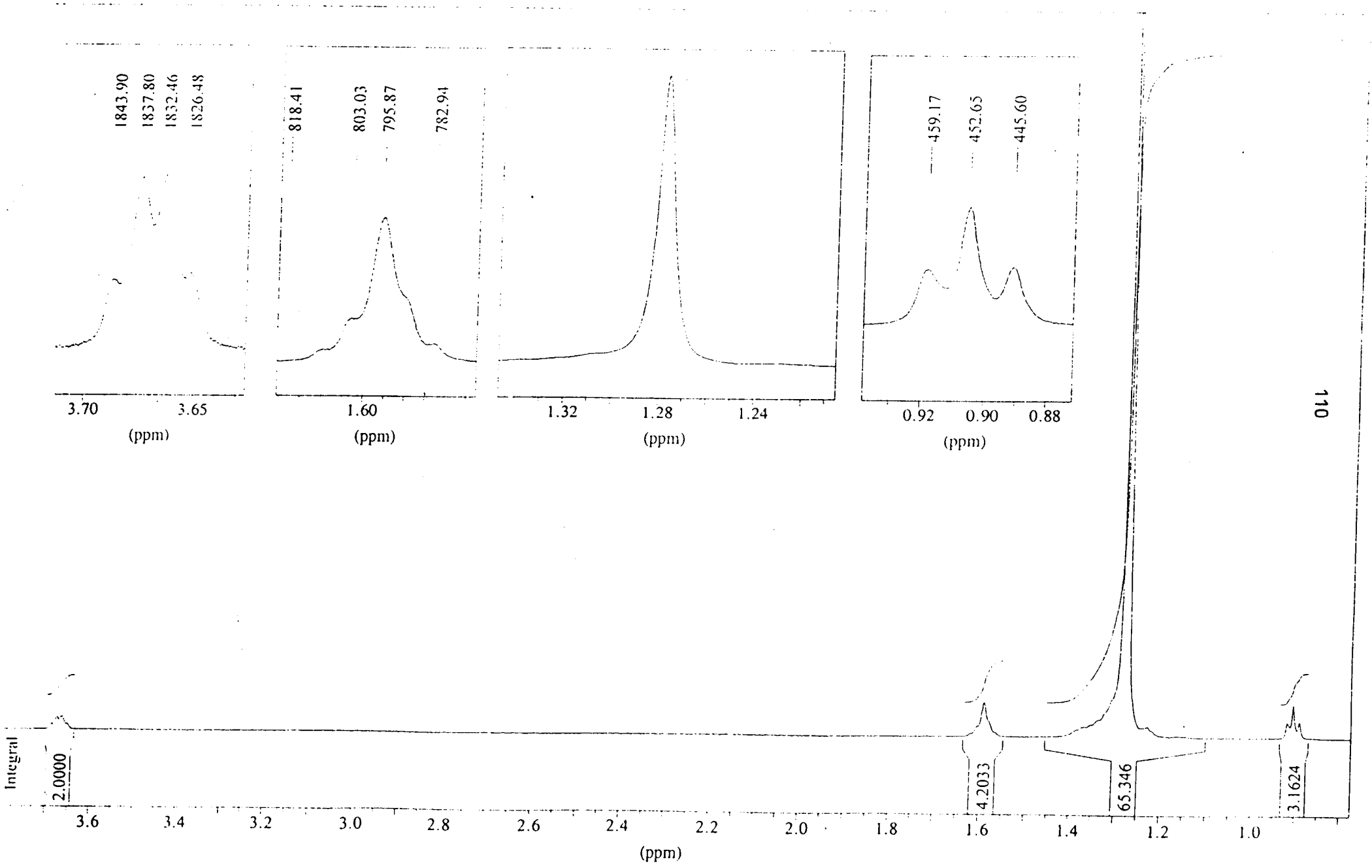
EI-mass spectrum of U_2 [Tritriacontane]

II.5.3. Characterization of U_3

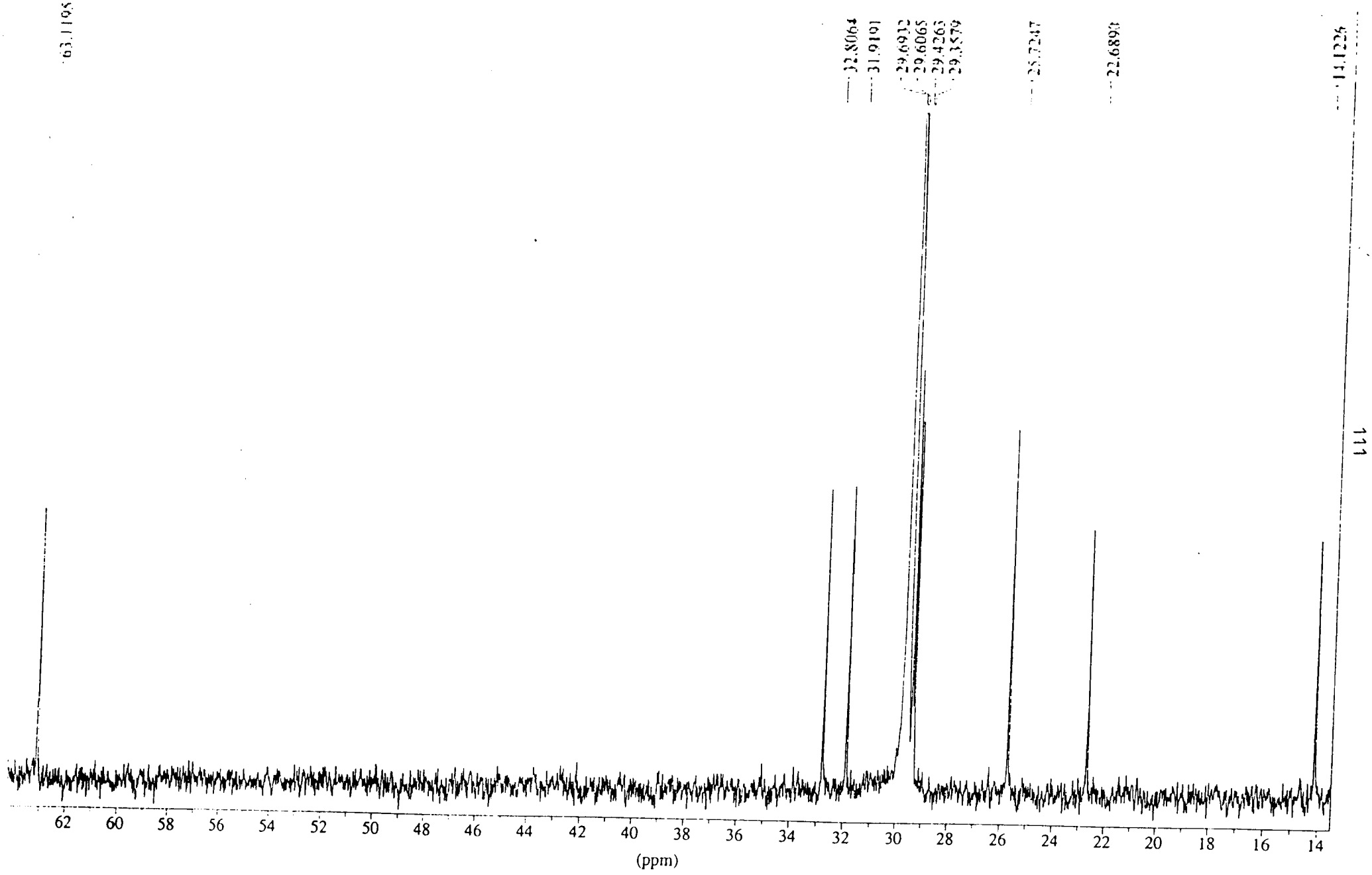
The component U_3 isolated from the petroleum ether extract on elution with 4:1 benzene-ethyl acetate as described under section II.4.2. as a white powdery substance (400 mg) melted at 86°C. It answered negatively towards Libermann-Burchard reagent for triterpenoids and sterols. The IR Spectrum indicated the presence of a hydroxyl group (Broad absorption with maximum at 3451 cm^{-1} (O-H stretching) and 1063 cm^{-1} (C-O stretching). Absorptions at 732 cm^{-1} and 718.5 cm^{-1} showed its long chain hydrocarbon nature. The presence of a primary hydroxyl group is also evident from the ^1H NMR absorption at δ 3.68 corresponding to two protons which is supported by the ^{13}C NMR peak at δ 63.12 ($-CH_2-OH$). The high resolution mass spectrum of U_3 had m/z 476.4 as the highest mass peak. This corresponds to $(M-18)^+$ peak of tetratriacontanol ($C_34H_{69}OH$). Peaks at m/z 448.3, 420.3 and 392.3 do not corresponed to any fragment ions. But it can be due to the $(M-18)^+$ ions of $C_{32}H_{65}OH$, $C_{30}H_{61}OH$ and $C_{28}H_{57}OH$. The melting point of U_3 supports this conclusion in that its m.p. was found to be 86°C which is lower than the reported m.p. (92°C)[235] of tetratriacontanol. Hence it is concluded that U_3 was a mixture of four n-alkanols $C_{34}H_{69}OH$, $C_{32}H_{65}OH$, $C_{30}H_{61}OH$ and $C_{28}H_{57}OH$.



High resolution mass spectrum of U_3 [Mixture of alkanols]



^{1}H-NMR spectra of U_3 [Mixture of alkanols] (500 MHz, $CDCl_3$, TMS)



^{13}C-NMR spectrum of U_3 [Mixture of alkanols] (125 MHz, $CDCl_3$, TMS]

II.5.4. Characterization of U_4

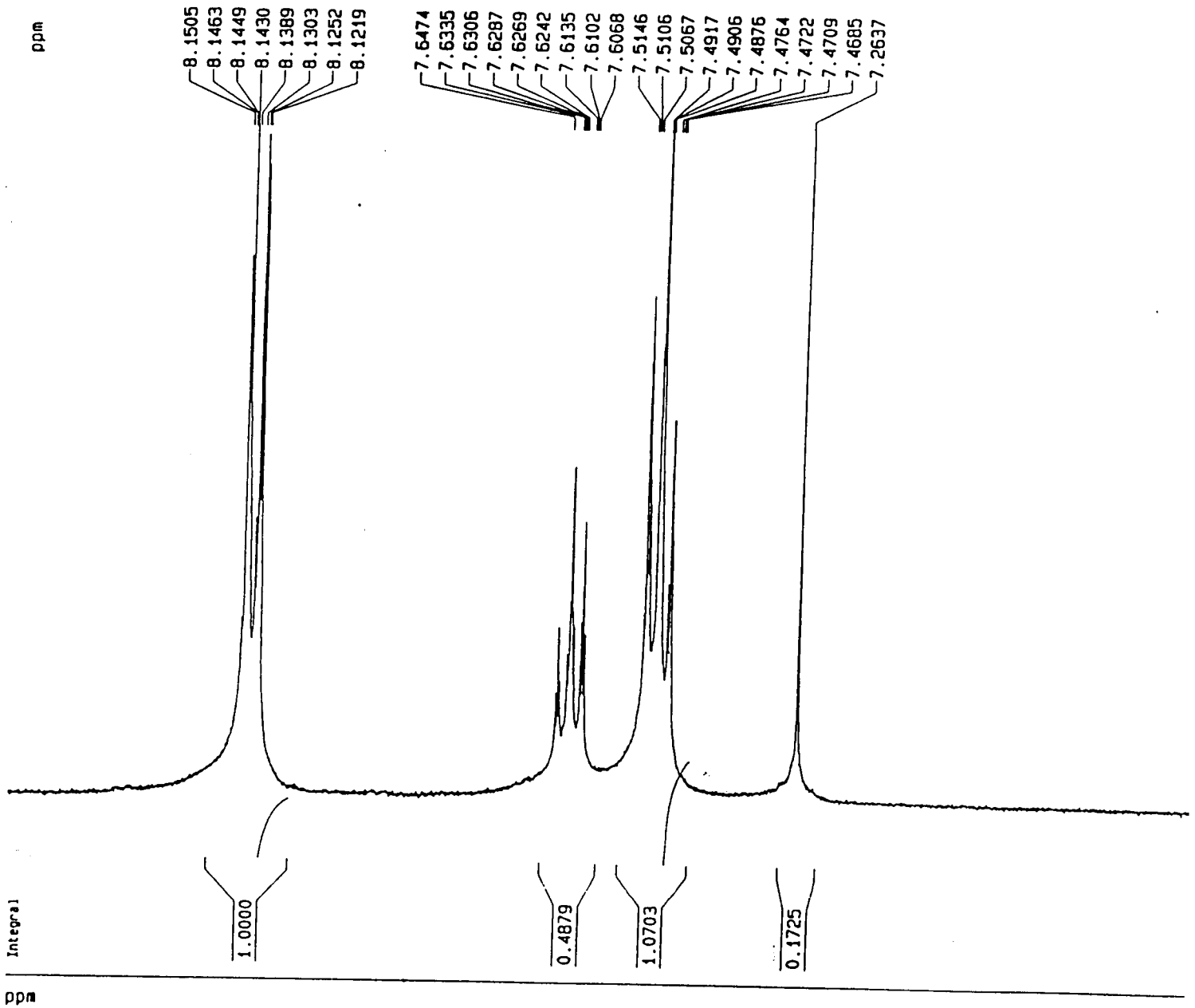
Compound U_4 was isolated from the petroleum ether extract on elution with 3:1 benzene-ethyl acetate as described in the section II.4.2. It was obtained as white shining needles and had m.p. 137°C and gave a play of colours with liebermann-Burchard reagent, indicating it to be a sterol. EI Mass spectrum of U_4 had M^+ at m/z 414 and base peak at m/z 55. Fragmentation pattern was quite comparable with that of β-sitosterol. IR Spectrum showed the presence of a hydroxyl group (broad absorption at 3457 cm^{-1}). Bands due to gem dimethyl group (doublet at 1386.1 and 1384.3 cm^{-1}), -CH stretching and bending bonds (298.3, 2821, 1460, 1470 cm^{-1}) were also observed in the spectrum. M.P, R_f (0.33 in benzene) and spectral data of U_4 were quite consistent with that of β-sitosterol. The acetate (m.p. 125°C) and benzoate (m.p. 144°C) of this compound were prepared and found to be identical with β-sitostorl acetate (m.p. 127°C) and benzoate (m.p. 145.5°C).[131] The procedure followed for their preparation is exactly as in chapter I.

II.5.5. Characterization of U_5

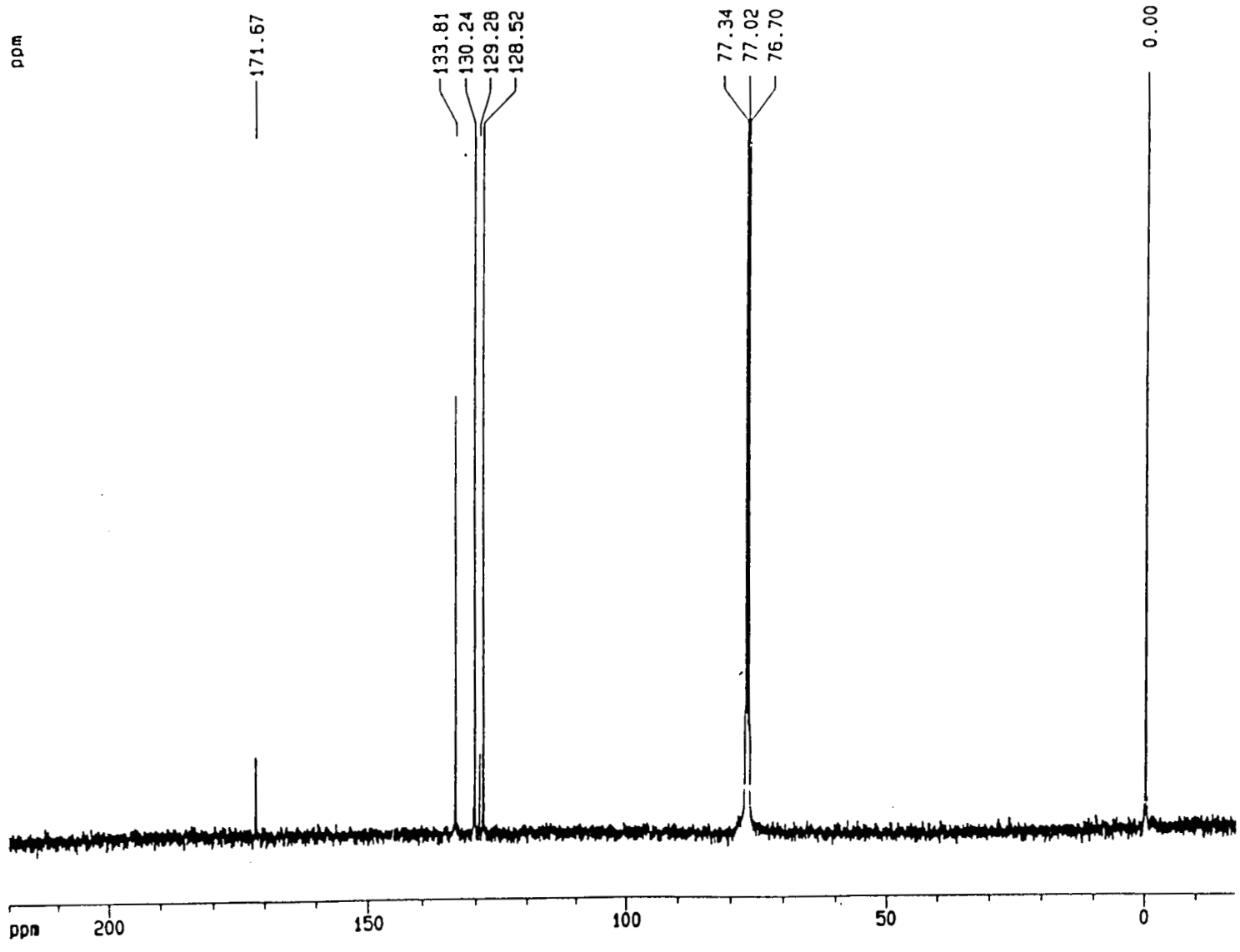
U_5 was obtained by fractionation of alcohol extract with benzene as described in section II.4.3. Melting point of this compound was found to be 119°C. On spraying with H_2SO_4 and heating it gave no colour. It also didnot answer

Liebermann-Burchard reaction. Absroptions and splitting patterns in ^{1}H NMR [δ 7.5 (t,2H,J = 5Hz), δ 7.6 (t,1H,J = 5Hz), δ 8.14 (d,2H,J = 5Hz)], suggested the presence of a monosubstituted benzene ring. The five peaks in the ^{13}C NMR spectrum, [4 aromatic (128.52, 129.28, 130.24 and 133.81) and one carbonyl (171.67)] further confirmed the presence of a monosubstituted aromatic compound. The APT spectrum also was in conformity with this having three -ve peaks (carbons with odd number of protons) and two +ve peaks (carbons with no protons bonded to it). IR Spectrum of U_5 showed a broad absorption in the region 2559.1-3073 cm^{-1}, indicating carboxyl -OH group. IR Spectrum also showed an intense absorption band at 1686.0 cm^{-1} showing the presence of a conjugated C = O group. Compound U_5 also gave brisk effervescence with $NaHCO_3$ solution, confirming the presence of a carboxyl group. Melting point and all spectral data confirmed the compound U_5 as benzoic acid.

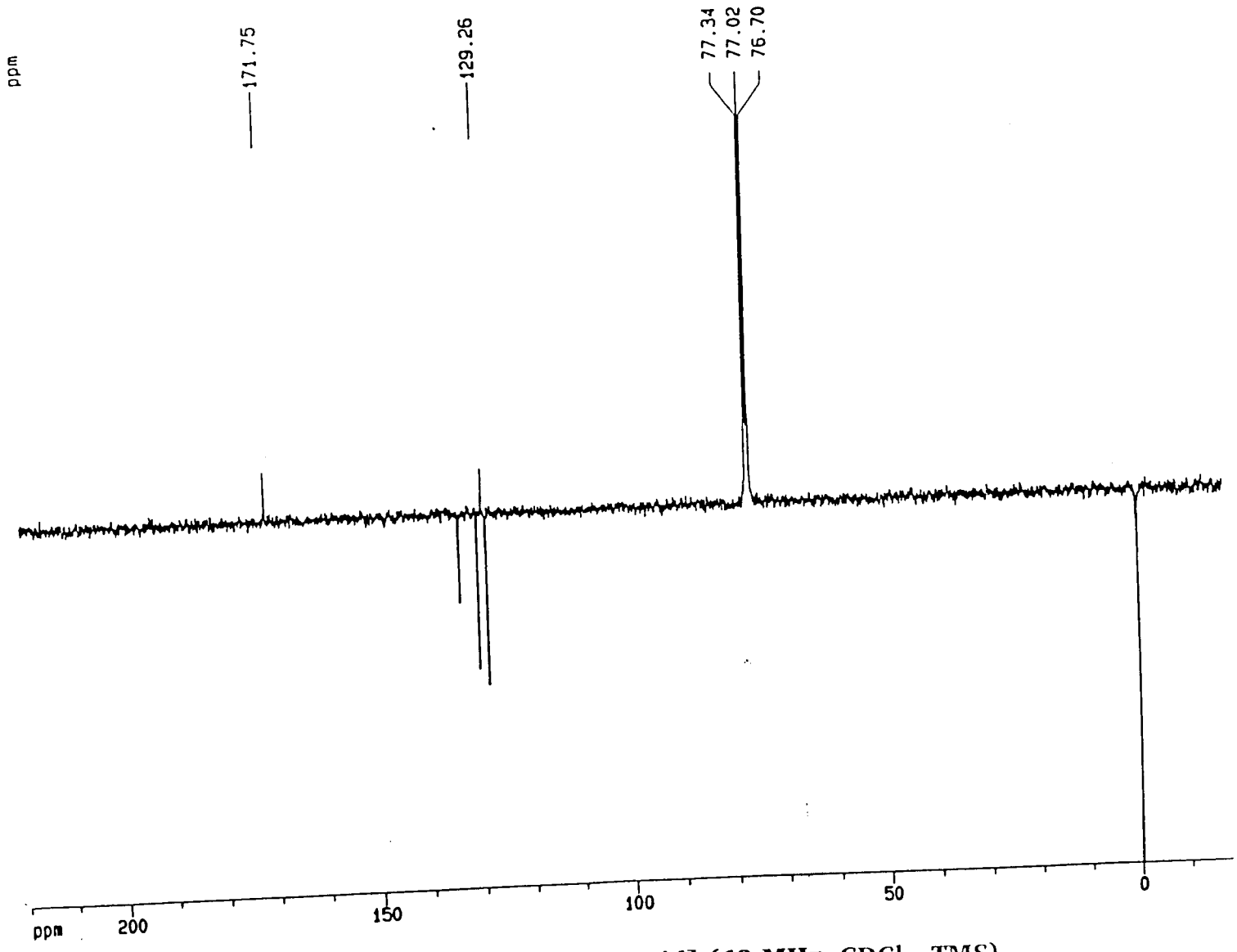
The amide of U_5 was prepared by heating it (0.1g) with 0.5 mL of redistilled thionyl chloride in a dry test tube over a steam bath for five minutes and adding liquor ammonia (5 mL) solution to it. The contents were transferred into a beaker and evaporated to dryness by gentle heating. The solid product obtained on recrystallisation from water melted at 128°C which was identical with the reported m.p. (129°C) for benzamide.[238]



^{1}H-NMR spectrum of U_5 [Benzoic acid] (250 MHz, $CDCl_3$, TMS)



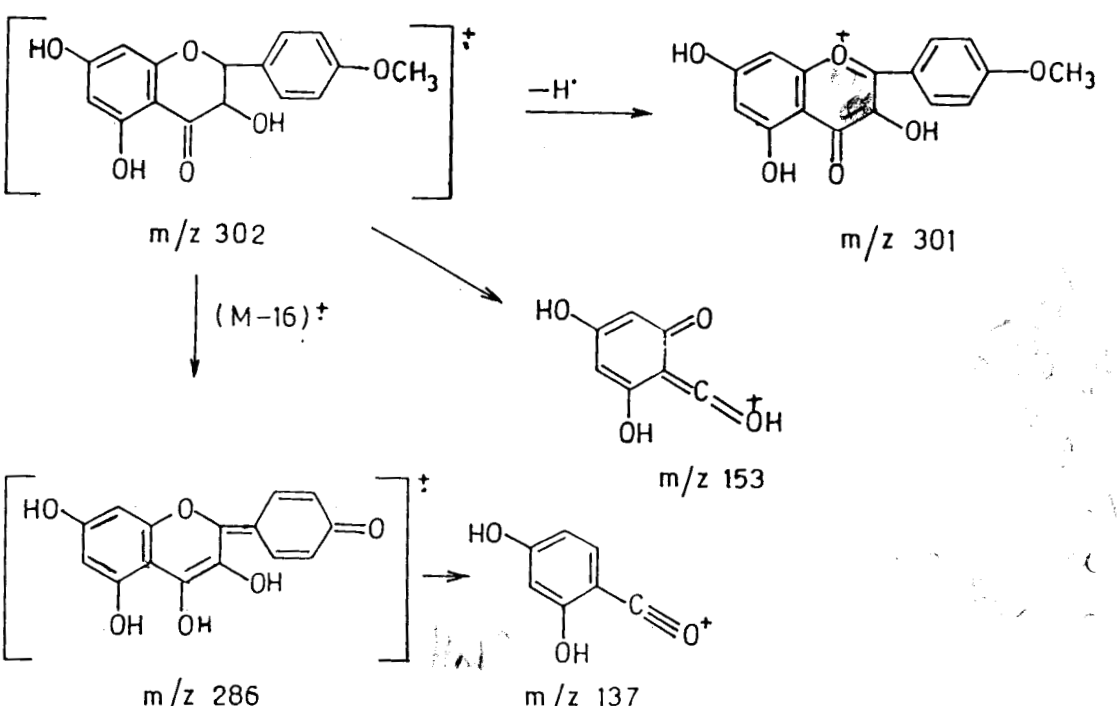
^{13}C NMR spectrum of U_5 [Benzoic acid] (63 MHz, $CDCl_3$, TMS)

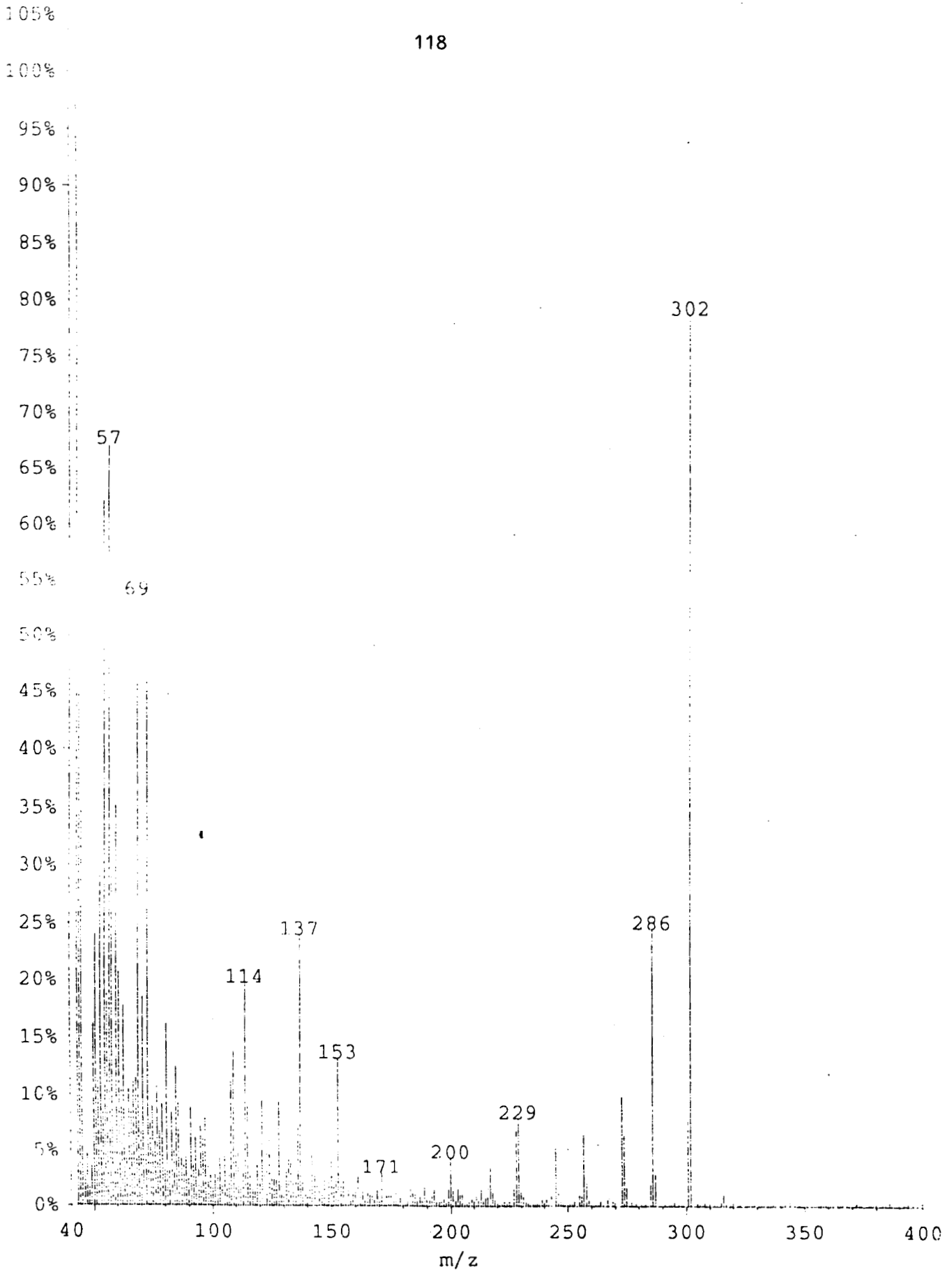


APT-^{13}C of U_5 [Benzoic acid] (63 MHz, $CDCl_3$, TMS)

II.5.6. Characterization of U_6

U_6 was isolated from the ethyl acetate fraction of alcohol extract as described in section II.4.3. It was obtained as yellow shining crystals (2 mg). This compound gave a yellow spot on TLC, colour being intensified on spraying with 5% $AlCl_3$, or fuming with NH_3, and brown colour under ultraviolet light. EI Mass sepctrum of this compound showed a very intense peak at m/z 302. This was the base peak and molecular ion peak. Other prominent peaks were at m/z 286, 273, 257, 229, 171, 153 and 137. This fragmentation pattern given below is comparable with that of a flavonol, dihydrokaempferol 4'-methyl ether.[239] Due to paucity of the material, further analysis couldn't be conducted to confirm the identity.





EI-mass spectrum of U_6 [Dihydrokaempferol 4'-methyl ether]

Parmar and coworkers[235] had reported the isolation of tritriacontane alone from this plant. In contrast to their finding this work found the presence of $C_{29}H_{60}$, $C_{30}H_{62}$ and $C_{31}H_{64}$ along with tritriacontane ($C_{33}H_{68}$). They also reported the isolation of tetratriacontanol. But the melting point reported by them (86-87°C) is lower than the reported value (92°C).[235] From the high resolution mass spectrum of U_3 it could be concluded that the component isolated by them can only be a mixture of alkanols and not tetratriacontanol as reported by them.

ANALYSIS OF UVARIA NARUM LEAF ESSENTIAL OIL

Bindu. T. K “Phytochemical investigation of a few plants” Thesis. Department of Chemistry, University of Calicut, 1998

CHAPTER III

ANALYSIS OF UVARIA NARUM LEAF ESSENTIAL OIL

III. 1. Introduction

An essential oil is the volatile fraction obtained from a plant or plant part by a physical separation method like water, steam or dry distillation, expression etc. Most part of these oils are made up of low molecular weight organic molecules of carbon, hydrogen, oxygen and occasionally nitrogen and sulphur. Essential oils usually contain a large number of components. By far the largest class of natural volatiles of plants is the terpenes. Modern analytical methods are capable of separating and identifying these large number of components in them. With the advent of capillary gas chromatography (with the more sensitive flame ionisation detector-FID), gas chromatography - mass spectrometry (GC-MS) and gas chromatography - infrared spectroscopy - mass spectrometry (GC-IR-MS), separation and identification of trace components have become possible. The olfactoric properties of essential oil components can be evaluated by gas chromatography olfactometry, more commonly referred to as GC-Sniffing. Provided that the GC conditions adequately separate the components of a mixture, each

component can be smelt in an olfactorily pure state at the exit port of a GC column.

Literature survey revealed that no study has so far been carried out on the leaf essential oil of Uvaria narum. More over essential oils obtained from plants belonging to annonaceae family have been used in perfumery[240]. Hence, analysis of the leaf essential oil of this plant was aimed at identifying the components present and also to evaluate the olfactoric property of different fractions.

III.2. MATERIALS AND METHODS

Uvaria narum leaves were collected from the Calicut University Campus. The shade dried leaves (800 g) were powdered in an electric mixer grinder and subjected to steam distillation for 5h. The distillate was extracted with solvent ether (3 x 100 mL). The ether portions thus obtained were pooled together and dried with anhydrous sodium sulphate. On evapouration of the dry ether extract after removal of sodium sulphate, over a water bath yielded 0.9 g (0.11%) of the essential oil.

Gas chromatographic analysis of the volatiles of the leaf essential oil was carried out using Shimadzu 14A (FID) gas chromatograph with hydrogen as carrier gas. For GC-MS,

a Shimadzu 17A GC-QP 5000 instrument with helium as carrier gas was employed. The mass spectra were recorded in the 35-450 amu range (EI mode, 70 eV). Hewlett-Packard GC/IRD/MSD instrument with IR range of 4000-850 cm^{-1} (MCT detector) was used for the GC-IR-MS analysis, 35-450 amu being the MS range. Columns for all the above analysis were 30 m x 0.32 mm bonded FSOT-RSL-200 fused silica (film thickness: 0.25 um, Biorad Co.) and 30 m x 0.32 mm bonded stabil wax fused silica (film thickness 0.5 µm, Restek Co). For GC-sniffing Carlo Erba GC-2101 with split system and FID was used. The column for GC-sniffing was 30 m x 0.53 mm mega bore FSOT-RSL-200 (1.0 µm film thickness, Biorad Co.). The temperature programming for GC, GC-MS, GC-IR-MS and GC-sniffing were the same. The sample was kept at 40^{o} for 5 min. and then heated to $250^{o}C$ at a rate of $10^{o}C$/min.

III.3. RESULTS AND DISCUSSION

a) Identification of components

The gas chromatogram of U. narum leaf oil showed the presence of about 100 compounds. Out of these sixty six were identified by comparison of their mass spectral and infra red spectral data with those in the computer database of the instruments. The retention indices of the identified compounds matched very well with the published data.[241, 242]

The identified compounds include fourteen monoterpenes, twenty five sesquiterpenes nine hexane derivatives and nine aromatic compounds. The main constitents (conc. higher than 4%) of the leaf oil were β-caryophyllene (9.99%), benzoic acid (9.75%), benzyl benzoate (6.23%), α-bulnesene (5.74%), β-elemene (5.61%), α-Copaene (5.39%), 3-hexenoic acid (4.46%) and germacrene-D (4.26%). All the identified compounds and their percentages are listed in table III.I and the structures of terpenoids are also provided.

Table. III.I Chemical composition of leaf essential oil of *Uvaria narum*

Compound	Percentage
Monoterpenes	
Borneol	1.22
Bornyl acetate	2.47
Camphene	0.23
Camphor	0.11
α-Fenchyl alcohol	0.33
Geraniol	0.36
Limonene	1.02
Linalool	0.78

Myrcene	0.25
trans-β-Ocimene	0.09
α-Pinene	0.31
β-Pinene	0.19
γ-Terpinene	0.21
α-Terpineol	0.56
Sesquiterpenes	
allo-Aromadendrene	0.39
β-Bisabolol	1.59
α-Bulnesene	5.74
γ-Cadinene	1.20
β-Caryophyllene	9.99
Caryophyllene oxide	2.81
α-Copaene	5.39
α-Cubebene	1.14
β-Cubebene	0.42
Cyclosativene	0.98
β-Elemene	5.61
δ-Elemene	2.17
β-Farnesol	1.23
trans-β-Farnesene	2.31
Germacrene-D	4.26
α-Guaiene	2.04
α-Gurjunene	2.03
α-Humulene	1.88

Longifolene	1.22
α-Muurolene	5.74
β-Patchoulene	1.28
Patchoulenone	1.15
β-Selinene	0.91
Spathulenol	1.18
α-Ylangene	0.41
<u>Hexane derivatives</u>	
Hexanoic acid	1.17
Hexanol	0.83
Hexen-3-ol	2.31
3-Hexenoic acid	0.51
3-Hexenol	1.82
3-Hexenyl acetate	0.42
3-Hexenyl benzoate	1.32
Hexyl acetate	1.43
Hexyl benzoate	0.66
<u>Aromatic compounds</u>	
5-Benzofuranyl acetate	0.33
Benzoic acid	9.75
Benzopyran	0.05
Benzyl acetate	0.54

Benzyl alcohol	1.12
Benzyl benzoate	6.23
2-Phenylethanol	0.51
2-Phenylethyl acetate	0.46
Salicyl benzoate	1.12
Others	
2-Dodecanal	0.26
Indole	0.31
Nonen-4-ol	0.21
Octanol	0.62
Octanoic acid	1.17
1-Octen-3-ol	0.73
Phytol	0.10
4-Undecanol	0.19

The high concentration of sesquiterpenic compounds (about 60%) is remarkable as the identification of about 10% aromatic compounds. The presence of aromatic compounds in U. narum has so far been reported by only one group[235]. Benzyl benzoate and its derivatives as well as other aromatic compounds were reported to be present in Uvaria species.[154, 156, 157] However, 5-benzofuranyl acetate and benzopyran are new aromatic constituents of this species.

The leaf essential oil of U. narum was found to be highly inhibitory to the growth of Phytophthora capsici the foot rot pathogen of pepper (chapter V), while the root essential oil inhibited the growth of the same plant pathogen to a very low extent. Hence the presence of aromatic constituents in the leaf oil and their absence in the root oil (chapter IV) was the lead to the finding that benzoic acid is quite inhibitory to the growth of P. capsici. Benzyl benzoate also was found to have this property to a lesser degree.

b. Olfactoric properties

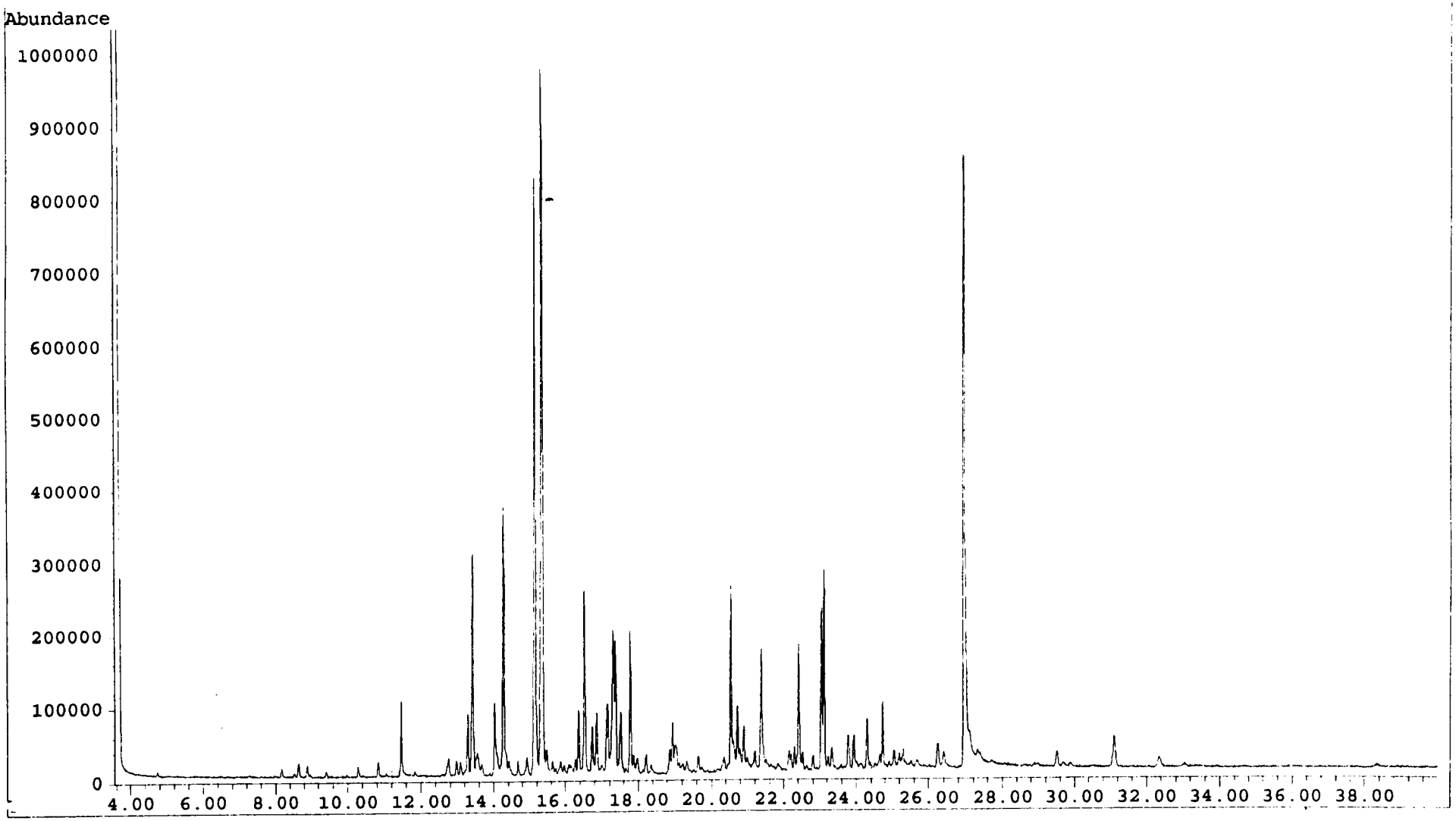
The genuine essential oil sample on olfactoric analysis revealed its odour to be unpleasant herbal-green when smelled in high concentrations. When diluted, it was described as being alcohol, dry fruity (hay, dried plums), damascone-like, osmanthus-like, herbal-spicy (lovage-like), possessing earthy and tobacco-like side notes with fatty and sweat-like sensations. Using a GC-sniffing technique, it was possible to determine that the sesquiterpenes were responsible for the herbal (in high concentration unpleasant), green, spicy, earthy and tobacco-like odors. The monoterpenes and aromatic compounds were found to be responsible for the fruity, damascone and osmanthus-like

notes, where as the hexane derivatives were responsible for the earthy, fatty and sweat-like odor impressions.

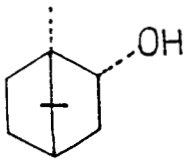
Aroma chemicals usually are hydrophobic organic compounds containing a limited number of functional groups. However the presence of a functional group is not a pre-requisite for odour.[243] Aroma chemicals have two sensory odour properties; intensity (strong, moderate, weak), quality (floral, woody etc.). Odour descriptions from two laboratories may be slightly different due to the absence of a universal odour language and the subjective nature of odour perception. The common scheme employed for the broad grouping of odours is based on our association with natural products. Thus materials with smell of roses, lily-of-the-valley, jasmine etc. are all placed in the floral family.

In the present olfactoric study the sesquiterpenes were found to be responsible for the herbal, green, spicy, earthy and tobacco-like odor. But at higher concentration, the odour is unpleasant. This observation is not unusual as similar examples are reported. Thiols at high concentrations exhibit unpleasant and sulphurous odours but at very low concentrations can have very pleasant grape fruit, pineapple or passion fruit notes. For example, p-menth-1-ene-8- thiol which is one of the most potent

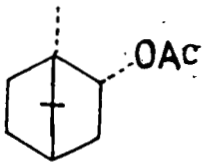
odorents known, has a characteristic grape fruit note at parts per billion (ppb) level or below, where as concentrated samples smell extremely powerful and nouseous[244]. The fruity damascone and osmanthus-like notes of U. narum leaf essential oil were attributed to the presence of monoterpenes and aromatic compounds. It is known[245] that benzyl esters have the odour of peach while apricot odour is similar to those of benzyl acetate and phenylethyl acetate. These compounds were present in this essential oil. The octane and nonane derivatives were responsible for the earthy, fatty and sweat-like odour impressions, where as the hexane derivatives were responsible for the green notes. Green note is an odour which is reminiscent of green foliage such as leaves, stalks and green vegetables and the smell of freshly cut grass. The compound which is released when a lawn is mowed is cis-3-hexen-1-ol.[243] The green odour of the trans-isomer is less pronounced. Generally hexane derivatives impart this odour. The leaf essential oil of U. narum contains nine hexane derivatives including 3-hexen-1-ol.



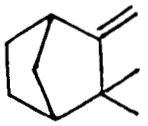
Gas chromatogram of *Uvaria narum* leaf essential oil



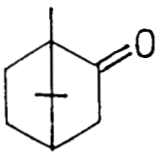
Borneol



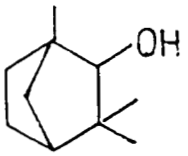
Bornyl acetate



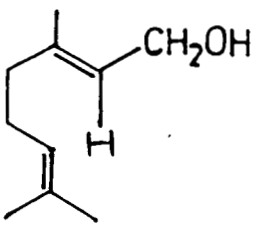
Camphene



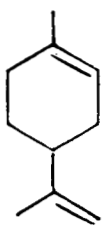
Camphor



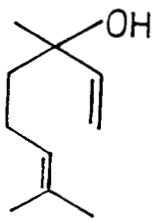
Fenchol



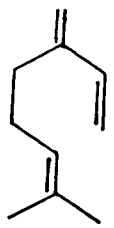
Geraniol



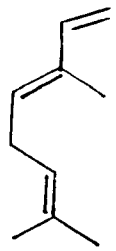
Limonene



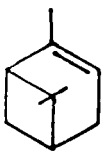
Linalooi



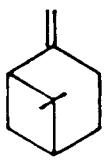
Myrcene



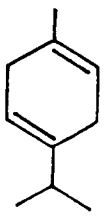
(E)·β-Ocimene



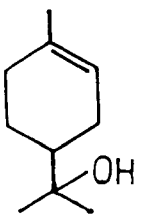
α-Pinene



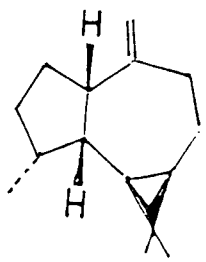
β-Pinene



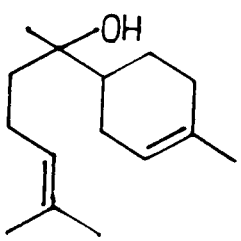
γ-Terpinene



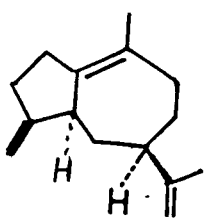
α- Terpineol



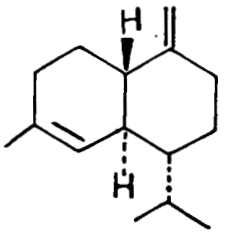
allo-Aromadendrene



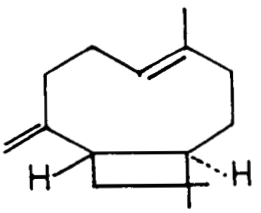
β-Bisabolol



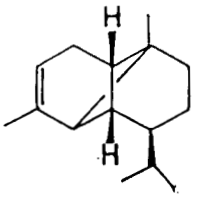
α-Bulnesene



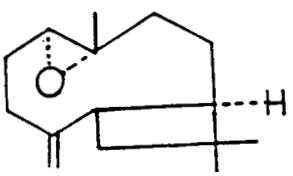
γ-Cadinene



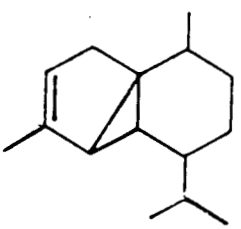
β-Caryophyllene



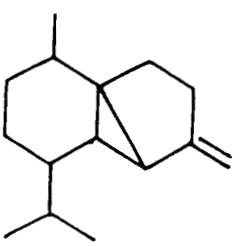
α-Copaene



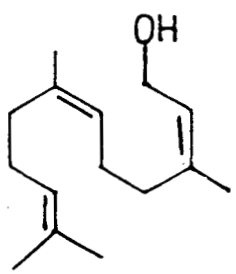
Caryophyllene oxide



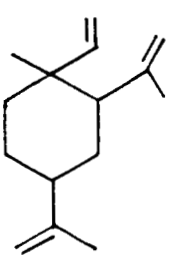
α-Cubebene



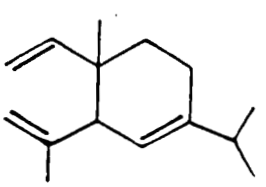
β-Cubebene



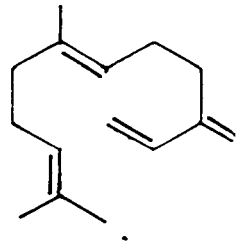
β-Farnesol



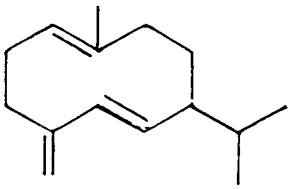
β-Elemene



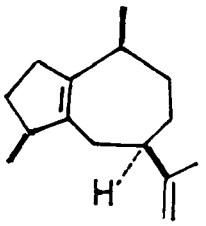
δ-Elemene



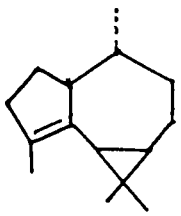
β-Farnesene



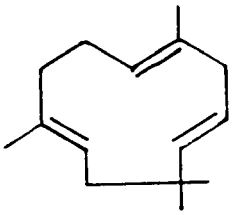
Germacrene-D



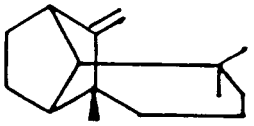
α-Guaiene



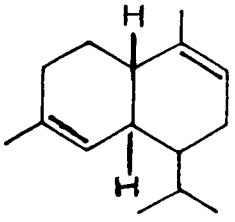
α-Gurjunene



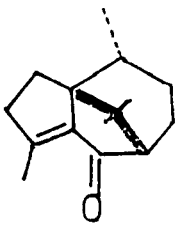
α-Humulene



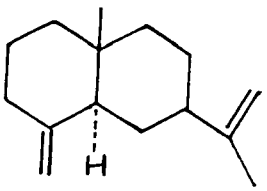
Longifolene



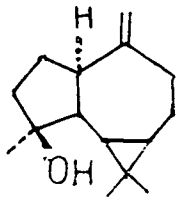
α- Muurolene



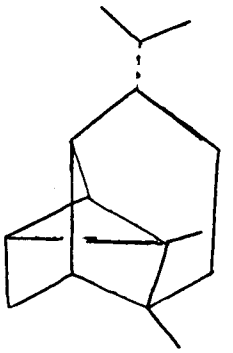
Patchoulenone



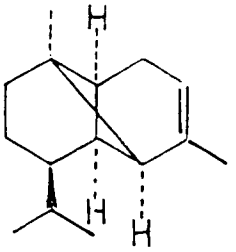
β-Selinene



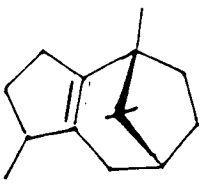
Spathulenol



Cyciosativene



α-Ylangene



β- Patchoulene

ANALYSIS OF ROOT BARK ESSENTIAL OIL OF UVARIA NARUM

Bindu. T. K “Phytochemical investigation of a few plants” Thesis. Department of Chemistry, University of Calicut, 1998

CHAPTER IV

ANALYSIS OF ROOT BARK ESSENTIAL OIL OF UVARIA NARUM

IV.I. Introduction

Already from ancient times, the essential oils have assumed considerable significance in the therapy of diseases as well as in food preparations as spices. Experts in the field of food sciences have recommended the utilisation of these 'aroma drugs' not only in dietetics, but also in other day-to-day home purposes. They are found to exhibit anti-inflammatory, antiseptic, deodorising, insecticidal or insect repellent, expectorating, appetite stimulating and sedative properties[246]. The antiseptic activity of essential oils has been known for a long time and many microbiological studies have been performed on essential oils. However, little is known about the mechanism of action of essential oils. Their lipoid solubility and therefore their possibility to penetrate into the cells may give rise to influence on the metabolism of the micro-organism and thus give an explanation of the effect. It has been observed[247] that the antiseptic activity of many compounds is dependent on their lipoid solubility. The antibacterial activity of essential oils has been expressed

in their phenol-coefficients. It tells us how many times stronger or weaker is the action of the essential oil in question, compared to phenol (phenol has the factor one).[246]

The root bark essential oil or the root bark as such of U. narum is used in traditional Indian medicine for erysipelas, eczema, fevers, biliousness and rheumatism[232]. In a previous study of this essential oil, it is reported to possess antifungal, antimicrobial, anthelmintic and central nervous system depressant activities[233]. Hisham, and coworkers[155] detected fifty two components in this essential oil but could identify only twenty five of them, while Nanda[233] and coworkers had identified five. Hence the aim of the present work was to identify as many compounds as possible and to discuss their part on known pharmacological activities. Of additional interest was the determination of volatiles responsible for the characteristic odour of this oil.

IV.2. MATERIALS AND METHODS

The roots of U. narum were collected from the Calicut University Campus. Shade dried root bark (250g) was powdered in an electric mixer grinder and subjected to steam distillation for 5 h. The distillate was extracted with

solvent ether (3 x 100 mL). The ether portion after drying with anhydrous sodium sulphate on evaporation gave 0.65 g (0.26%) of oil.

The volatiles of the root bark essential oil of U. narum were analysed by GC, Shimadzu 14 A (FID), with integrator, hydrogen being the carrier gas. For GC-MS a Shimadzu 17 A GC-QP 5000 instrument with helium as carrier gas was used. The mass spectra were recorded in the 35-450 amu range (EI mode, 70 eV). GC-FTIR-MS was carried out on Hewlett-Packard GC-IRD-MSD instrument with helium as carrier gas. IR Range used for the same was 4000-850 cm^{-1} (MCT defector) and MS range 35-450 amu. (EI mode, 70 eV).

GC-Sniffing technique was carried out using Carlo Erba GC with split system and FID. Columns for all measurements exclusively GC-sniffing technique was 30 m x 0.32 mm bonded FSOT-RSL-200 (film thickness; 0.25 μm, Biorad Co.) and 30 m x 0.32 mm bonded stabilwax fused silica (0.5 μm film thickness, Restek Co.). For GC-Sniffing technique the column used was 30 m x 0.53 mm mega bore FSOT-RSL-200 fused silica (1.0 um film thickness, Biorad Co.). The temperature programming for GC, GC-MS, GC-IR-MS and GC-sniffing were the same. The sample was kept at 40^{o} for 5 min. and then heated to $250^{o}C$ at a rate of $10^{o}C$/min. and splitless mode.

IV.3. RESULTS AND DISCUSSION

a) Identification of components

More than eightly compounds were detected by GC-FID analysis. Fifty seven of them could be identified by GC-MS and GC-FTIR-MS techniques by comparing their IR and MS data with those in the libraries of the respective instruments. Retention indices of the identified compounds were found to correspond to their reported values.[241,242]

Out of these fifty seven components identified, forty are being reported for the first time from the root bark essential oil of U. narum. The main constituents, (concentration higher than 4%) of the root essential oil were patchoulenone (24.5%), bornyl acetate (19.7%), borneol (8.6%), camphene (5.2%) and camphor (4.8%). In an earlier report[155] bornyl acetate was found to be the major constituent (15.2%) while patchoulenone was found to be only 8.1%. It is also worth mentioning that the root bark essential oil doesn't contain any aromatic compound while the leaf essential oil is rich in aromatic compounds. A comparison of leaf and root bark essential oils of U. narum and U. chamae shows that the leaf essential oil of U. narum contains nine aromatic compounds while the root bark essential oil contains none. In the case of U. chamae[154]

the leaf essential oil contains only one aromatic compound while the root bark essential oil contains seven. All the compounds identified are given in Table IV.1. The structure of terpenoids which are not present in the leaf essential oil are also provided.

Table IV.1 Identified compounds in the steam distilled root bark oil of Uvaria narum

Compound	Concentration*	n.d
Aromadendrene	0.87	+
Borneol	8.59	
Bornyl methyl ether	0.16	+
Bornyl acetate	19.66	
α-Bulnesene	1.47	+
α-Bulnesol	0.54	+
δ-Cadinene	1.83	+
δ-Cadinol	0.79	+
Camphene	5.21	+
Camphenhydrate	0.39	+
Camphenilone	0.16	+
Camphor	4.75	
δ-3-Carene	0.21	+
β-Caryophyllene	3.16	

β-Caryophyllenoxide	0.89	+
1,8-Cineole	2.91	+
α-Copaene	0.07	+
χ-Copaene	0.61	
p-Cymene	0.11	
Cyperene	1.01	
Decanol	0.89	+
Dibutylphthalate	0.71	+
α-Elemene	1.32	+
β-Elemene	0.42	
δ-Elemene	0.05	
Elemol	0.51	+
Fenchol	0.77	
Fenchyl acetate	1.29	+
Germacrene-D	0.43	+
α-Gurjunene	1.09	
Hexadecanoic acid	0.12	+
Hexanoic acid	0.44	+
1-Hexen-3-ol	0.74	+
α-Humulene	0.43	
2-Hydroxycineole	0.23	+
Isoborneol	3.18	+
Isobornyl acetate	0.14	+
Isocaryophyllene	0.82	

Limonene	0.17	
Linalool	0.61	+
Linalool oxide	0.16	+
Longifolene	0.06	+
α-Muurolene	0.61	+
Myrcene	1.09	+
β-Ocimene	0.02	+
Patchoulene	0.56	+
Patchoulenone	24.48	
Patchoulol	0.41	+
α-Pinene	0.34	+
β-Pinene	0.02	+
β-Selinene	0.52	
γ-Terpinene	0.22	+
Terpineol	1.69	
Tetradecanoic acid	0.61	+
Tricyclene	0.44	+
4-Thujenyl acetate	0.04	+
Verbenone	0.08	+

* Peak area percentage (GC-FID)

n.d: Until now not detectd in the root bark essential oil.

b) Olfactoric properties

The root bark essential oil was investigated by GC-

sniffing technique to get information about the components which were responsible for the characteristic odour impression of this sample, described as fresh, green, camphoraceous, borneol like, weak floral and in the back ground weak woody. The correlation substance-peak to odour property showed that camphane and borneol derivatives as well as some other monoterpenes like 1,8-cineole, fenchol and fenchyl acetate were responsible for the dominating fresh-camphoraceous-borneol-like odour notes.

Hexane derivatives, especially 1-hexen-3-ol exert green while patchoulene and pinene derivatives exert woody odour notes. The floral odour impression is a product of mono (e.g. linalool) and sesquiterpenes (e.g. δ-cadinol).

Green note exerted by this oil is attributed to hexane derivatives especially 1-hexen 3-ol. Hexane derivatives impart green odour, especially cis-3-hexen-1-iol.[245] However in this work, the specific isomer of 1-hexen-3-ol has not been identified.

The woody odour describes the odour of not only the essential oils derived from various trees such as cedarwood and sandalwood, but also some oils derived from leaves like patchouli and grass like Vetiver[245]. In this work, patchoulene, a constituent of patchouli oil and pinene

derivatives were found to elicit woody odour.

The floral odour is the combined contribution of mono and serquiterpenes. Of the monoterprnes, linalool which is present in this essential oil is the most prominent component which imparts floral odour. It is a constituent of rose, jasmine and neroli oil.[248]

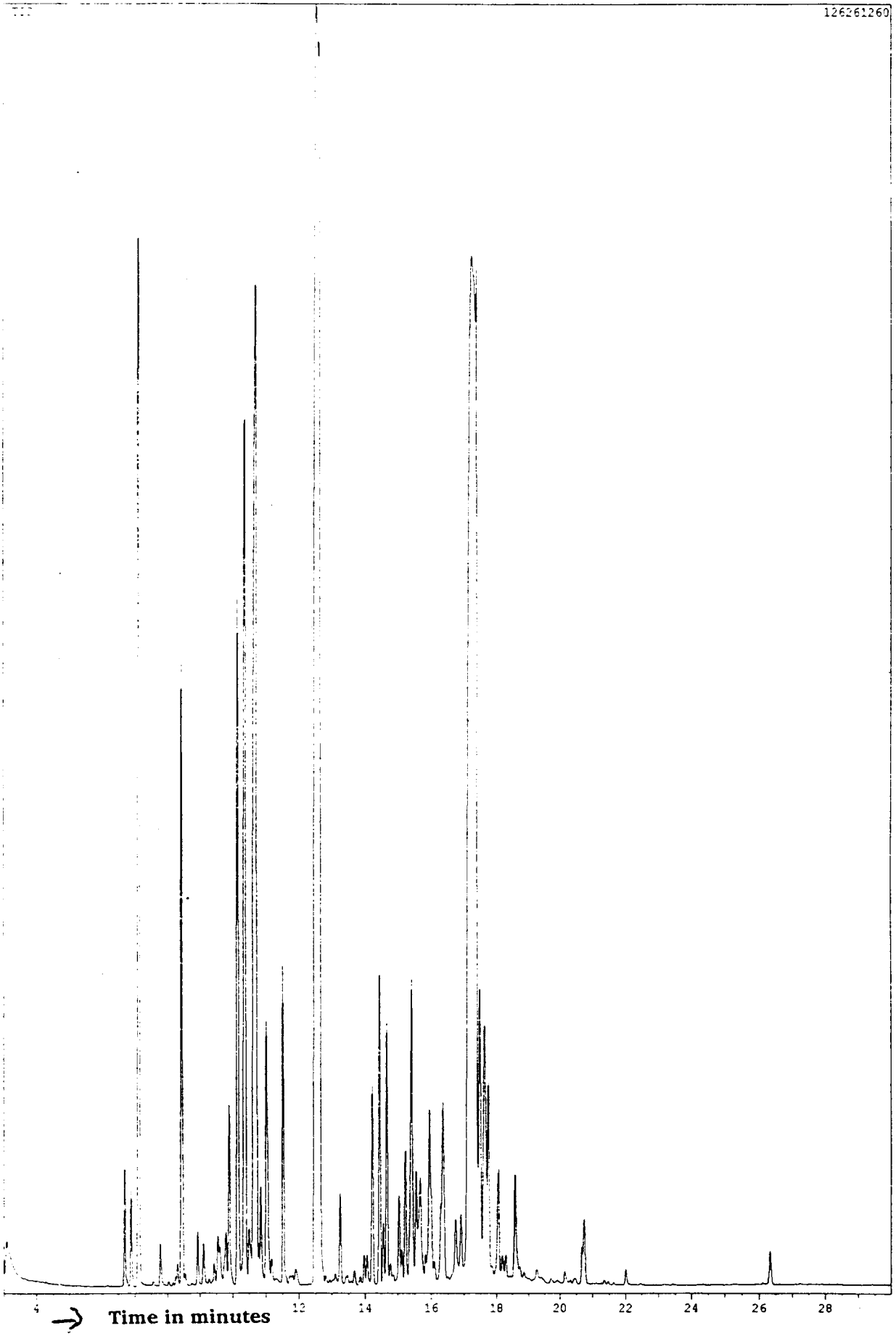
c) Pharmacological activity

J.C. Maruzzella and P.A. Henry investigated[249] the antimicrobiol activity of one hundred perfume oils on ten bacteria and ten fungi. All of them exhibited bactericidal or bacteriostatic and fungicidal or fungistatic activity. As perfumes contain volatiles the above mentioned study confirmed their antimicrobiol property.

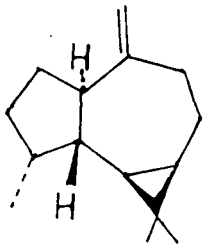
Terpenic compounds such as α-pinene are effective in promoting recovery from fatigue or diminishing the sense of fatigue.[250]

n-Decanol has been found to be active against the fungi Trichophyton mentagrophytes, T. rubrum and Epidermophyton floccosum which cause skin diseases. n-Decanol also exhibited bactericidal activity against Basillus subtiles and Staphylococcus aureus[251]. Phenol coefficients, an index of antibacterial activity, of linalool, α-terpineol and 1,8-

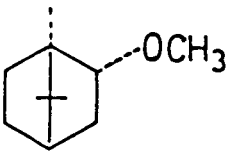
cineole are 5,4 and 2.2 respectively. All the compounds mentioned above are constituents of the root bark essential oil of U. narum. The medicinal property of this essential oil can thus be attributed to these compounds and probably to other less investigated compounds that are present in it. Thus the analysis of this oil used in Indian folk medicine as antibacterial, atifungal and anthelmintic drug allows the conclusion that not only the less volatile compounds, like acetogenin,[234] are responsible for the mentioned properties but also the volatiles.



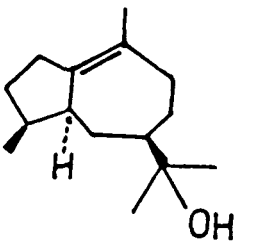
Gas chromatogram of *Uvaria narum* root bark essential oil



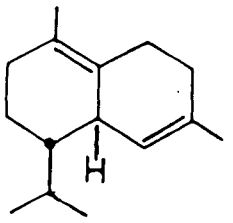
Aromadendrene



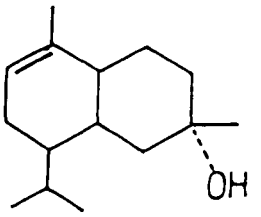
Bornyl methyl ether



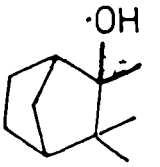
Bulnesol



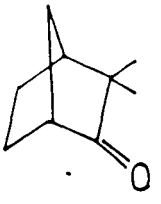
δ-Cadinene



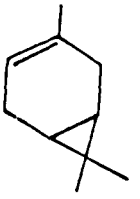
δ-Cadinol



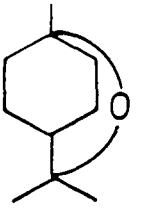
Camphenhydrate



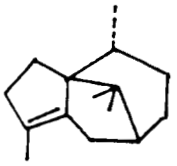
Camphenilone



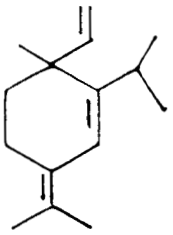
δ-3-Carene



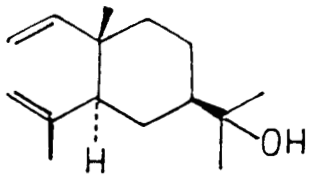
1,8-Cineole



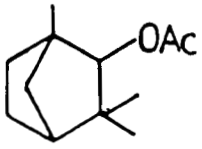
Cyperene



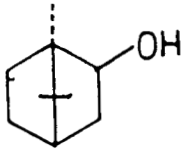
α-Elemene



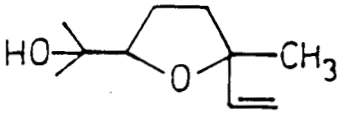
Elemol



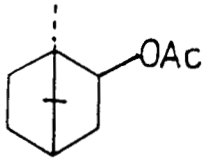
Fenchyl acetate



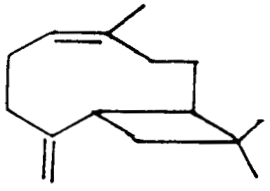
Isoborneol



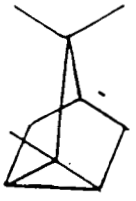
Linalool oxide (furanoide)



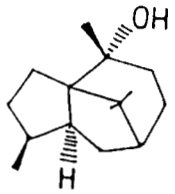
Isobornyl acetate



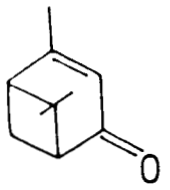
Isocaryophyllene



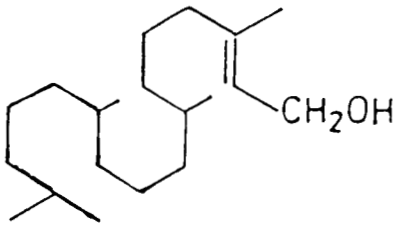
Tricyclene



Patchoulol



Verbenone



Phytol

SECTION 1: ANTIFUNGAL ACTIVITY OF UVARIA NARUM

SECTION 2: ANTIFUNGAL PROPERTY AND STRUCTURE-ACTIVITY RELATIONSHIP

Bindu. T. K "Phytochemical investigation of a few plants" Thesis. Department of Chemistry, University of Calicut, 1998

CHAPTER V

SECTION 1: ANTIFUNGAL ACTIVITY OF *UVARIA NARUM*

Introduction

Black pepper (*Piper nigrum* L) often referred to as king of spices is an important crop grown exclusively in humid tropics like India, Indonesia, Malaysia, Brazil, Thailand and Sri Lanka. In India, this spice accounts for considerable amount of earnings in foreign exchange (In 1996-97, it amounted to foreign exchange worth Rs.450 crores) and is cultivated in Kerala, Karnataka and Tamil Nadu. Besides insect pests and physiological disorders this crop is affected by many fungal, bacterial, viral, mycoplasmal and nematodal diseases. Among the fungal pathogens, *Phytophthora capsici*, earlier known as *Phytophthora palmivora* MF_4 is the most destructive one. It is responsible for the devastating disease 'foot rot' also known as 'quick wilt' of pepper causing severe crop loses in all black pepper producing countries.[252,253] The crop loss due to foot rot in Kerala is estimated to be 10% of the total production.[254]

Fungicide treatment is the primary means for

controlling fungal diseases of crops and this 'quick fix' mentality of agriculture is now changing. A number of fungicides are being withdrawn from the market either because of their ineffectiveness or as a result of the target pathogens developing resistance to them or for human health considerations. Public awareness of adverse effect of using synthetic fungicides has received interest in identifying materials from plants which would be toxic or antagonistic to plant pathogens. Fungicidal materials from plants are usually biodegradable and this is one of the most desirable characteristics for chemicals used for pest management. These natural fungicides not only provide leads to synthesise new structural type of fungicides that are relatively safe to man and his environment but also can help to meet the ever expensive supply of synthetic fungicides.

It is generally agreed that most, if not all, plants are resistant to majority of microorganisms in their environment. The presence of antimicrobial substances in higher plants is well-known since ancient times. Egyptians used mixtures of certain vegetable oils for the preservation of mummies from protein decomposing bacteria. Greeks and Romans used the juice of green walnut shells against fungal diseases of skin. Even today, clove oil is used to disinfect dental pulp cavities. However it is only

recently, intensive search to discover antimicrobial substances from higher plants which could be used for the control of diseases caused by plant pathogens is being made. This is of particular interest to India, because in Ayurveda, references are made to a large number of indigenous plants possessing antimicrobial properties.

Antifungal compounds in plants can be preformed in the plant [constitutive antifungal substances] or can be induced in the plant after infection [induced antifungal constituents or phytoalexins]. Constitutive antifungal compound can be either preinfectional or post infectional. Preinfectional inhibitors are compounds which are present in the plant before contact with the fungs has been established. They include catechol and protocatechuic acid present in the dead outer scales of coloured onion varities, removal of which renders it susceptible to attack by fungus. A true preinfectional inhibitor, 'beans factor', a ketoacetylenic ester is present in beans.[255] Postinfectional inhibitors are normal host metabolites which accumulate in higher concentration as a result of fungal attack. They include a large number of compounds like phenols, aromatic hydoxry acids etc. They are normally present in the plant in an inactive form but are converted into active antifungal compounds after infection by a simple

biochemical reaction such as enzymic hydrolyis.[256] For example cyanogenic glycosides release toxic HCN after infection or leaf damage. Phytoalexins on the other hand are post infectional inhibitors, whose production may take two or three days.

The fungi constitute a group of living organisms devoid of chlorophyll. They resemble simple plants in that with a few exceptions, they have definite cell walls. They are usually nonmotile, although they may have motile reporductive cells. Fungi reproduce by means of spores. A spore is a minute simple propagating unit without an embryo that serves in the production of new individuals of the same species. Fungi do not possess stem, roots or leaves as plants do have. They are usually filamentous and multicellular. The filaments constituting the body (soma) of a fungus elongate by apical growth. Reproductive structures are differentiated from somatic structures and exhibit a variety of forms on the basis of which fungi are classified.

The fungal thallus typically consists of microscopic threads or filaments that branch in all directions, spreading over or within the substratum utilized for food. Each of these filaments is known as hypha which is made of a

thin transparent tubular wall, filled with a layer of protoplasm, varying in thickness. The principal chemical constituents of most of the fungal cell wall are various poly saccharides, but proteins, lipids and other substances are also included. Chitin is characteristically present in the cell wall of most fungi. Oomycetes fungi are thought to be devoid of chitin and is reported to contain cellulose-β-glucan only.

The mass of hyphae constituting the thallus of a fungus is called mycelium. The mycelium of parasitic fungus grows profusely on the surface of or within the host spreading between the cells or penetrating into them. Fungi reproduce both sexually and asexually. In general asexual reproduction is more important for the propagation of the species because it results in the production of numerous individuals and particulary since the asexual cycle is repeated several times during the season where as the sexual reproduction takes place only once a year.

Asexual reproduction is a mode of propagation of new individuals such as simple division of a unicellular organism into daughter cells or of a multicellular thallus into a number of fragments each of which grows into a new individual. The most common way of asexual reproduction in fungi is by means of spores which vary in colour and shape.

Fungal spores produced asexually are either born in sporangia or are produced at the tips or sides of hyphae in various ways and are called conidia.

A sporangium is a sac-like structure whose entire contents are converted through cleavage into one or more, usually many spores. These spores may be motile or nonmotile. Motile spores are called zoospores which are having one or two flagella. If spores are nonmotile they are called aplanospores. The liberated zoospores swim about actively in a surface film of water for some time and then come to rest. Each quiescent zoospore retracts their flagella and may encyst. The encysted zoospores then germinate by putting out a special short hypha called germ tube. The germ tube then pierces the cuticle of leaf and then forces its way through the epidermis into the host tissue to bring about infection.

Phytophthora capsici comes under the class Oomycetes, which contain some most devastating plant pathogens. Classic examples of the diseases they cause include late blight of potato and downey mildews of different crops. Among Oomycetes, the Phytophthora spp. include plant pathogens causing an array of diseases ranging from foliar and fruit diseases to stem and root diseases.

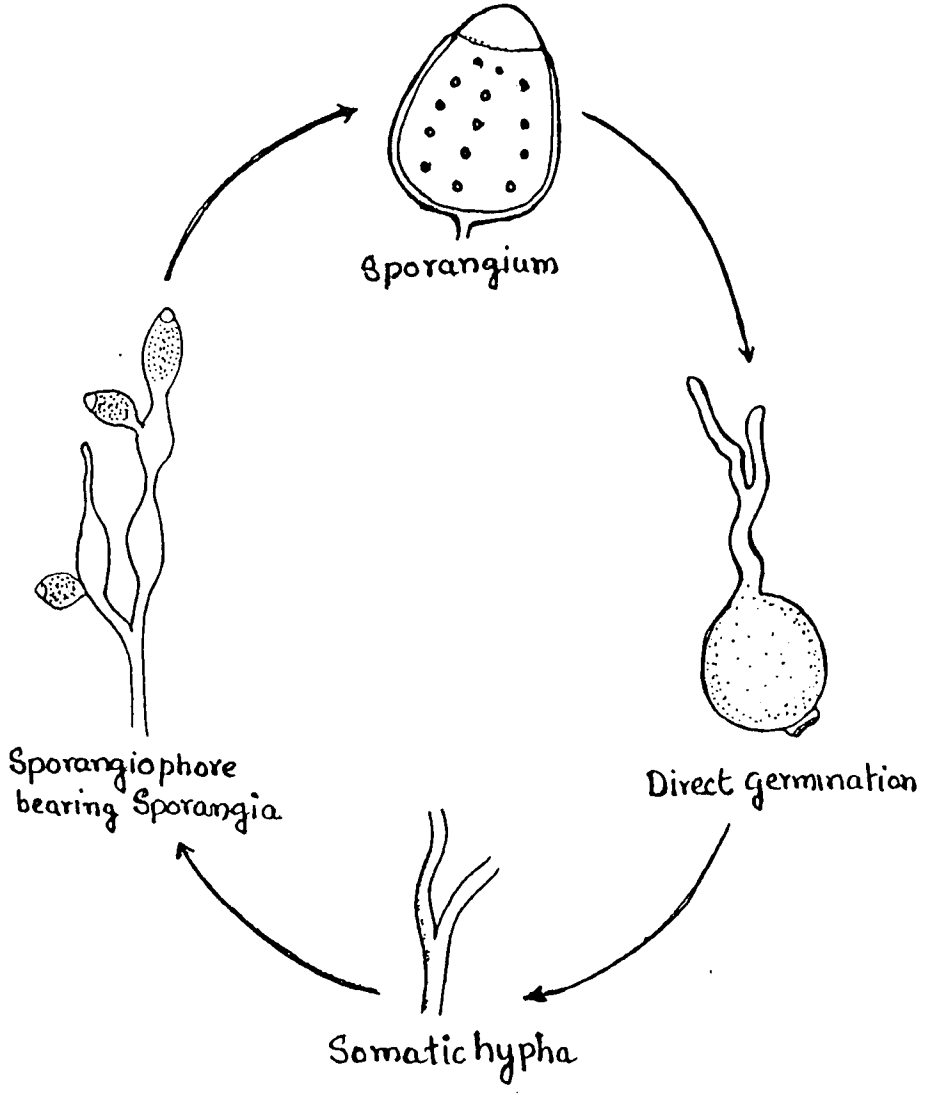
Phytophthora capsici affects all parts of the pepper vine. Its infection on underground parts namely roots and collar (foot) results in root rot and foot rot. Infection on the aerial parts like leaves, spikes and stems occur and spreads rapidly under favourable conditions causing defoliation and complete destruction of vines in severe cases. P. capsici is a wet weather pathogen and infection occurs mainly during south west monsoon (June-Sept.) period in Kerala. This fungus spreads mainly through rain splash and water.[257] High rainfall and microclimatic conditions like high relative humidity, low temperature and shorter duration of sunshine prevailing during this period are known to favour the increase in disease.[258] This fungus grows luxuriantly at 25-28^{o}C on carrot agar medium[259] and growth is absent at 35^{o}C. Sporangial formation is found to be maximum at pH 6 and absent at pH 3.

In P. capsici, spores are contained within the sporangium. Germination of this sporangium is governed by temperature and moisture. At low temperature (below 15^{o}C), and wet conditions, indirect germination occurs. In this case, cleavage of cytoplasmic contents results in the formation of uninucleate zoospores. Liberated zoospores swim in water film on the host, come to rest by withdrawing their flagella, get encysted and each produces a germ tube.

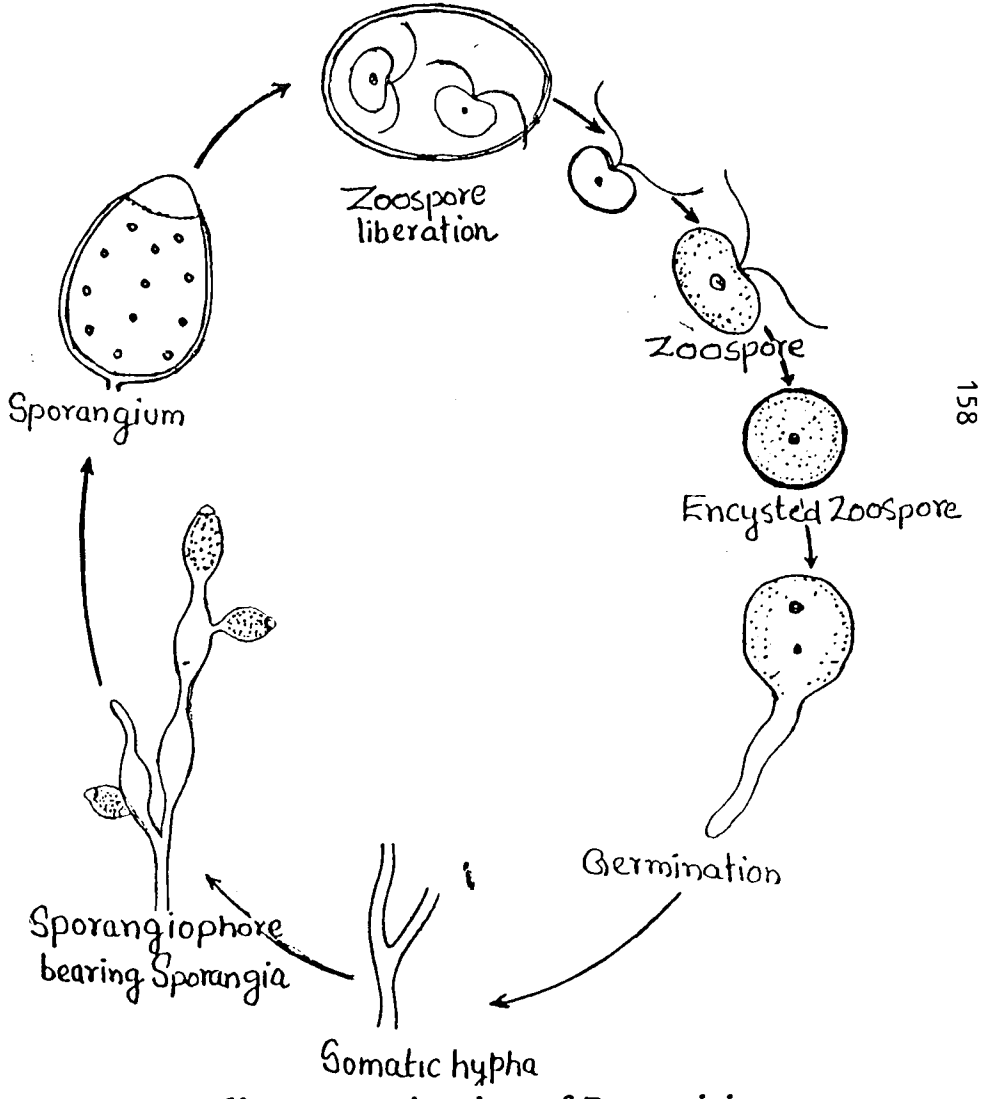
The germ tube penetrates to the host. The sudden disastrous effects of the phytophthora infection may be due to this indirect germination under favourable conditions.

High temperature and dry condition favour direct germination of sporangia. In this case sporangium behave as a single spore and germinates directly by forming a multinucleate germ tube. Zoospores are not formed. Optimum temperature for direct germination is 24^{o}C.

For a long time, the control of Oomycetes fungi remained a tough challenge to the plant pathologists. They were not amenable for the control with many earlier systemic fungicides as they are endowed with some unique biochemical and physiological features.



Direct germination of *P. capsici*



Indirect germination of *P. capsici*

Water solubility is considered to be the most important attribute of fungicides in order to be effective against Oomycetes which thrive in aqueous environments. For the control of 'foot rot' disease, a number of control measures involving cultural, chemical and biological have been advocated.[260] Chemical control measures include foliar spray and swabbing of the collar portions of the vines with Bordeaux mixture besides drenching of the soil with Bordeaux mixture or copper oxychloride and use of systemic fungicides like metalaxyl, fosetyl-Al. Biocontrol agents like Gleocladium virens, Trichoderma spp, Vesicular arbuscular mycorrhiza (VAM) are also used against this fungus. However the effect of botanical pesticides hasn't received enough attention. During recent years, use of plant secondary metabolites for the control of fungi is gaining importance.[261-263] The root exudates of Allium spp. have been reported to be inhibitory to P. capsici.[264] Inhibitory effect of ajoene, a component of garlic on P. drechsleri has been reported recently.[265] Soil application of a mixture of garlic and mustard has been reported to be effective against foot rot of black pepper.[266] Water and ethanol extracts of Piper colubrinum and Chromolaena odorata have been found inhibitory to P. capsici.[267]

A preliminary examination of the activity of Uvaria

narum leaf extract on the growth of P. capsici showed high inhibition. Hence it was proposed to investigate its effect in detail. It was also aimed at isolation and structural elucidation of the compounds responsible for inhibitory activity against P. capsici.

MATERIALS AND METHODS

Root bark and leaf extracts were prepared from the materials collected from Calicut University Campus. The dried and powdered material (100 g each) was heated under reflux with sterile double distilled water (600 mL). After keeping overnight, they were filtered and the volume reduced to 100 mL by evaporation.

The P. capsici cultures for inoculation were prepared by growing them in carrot agar medium. Discs of 0.5 cm diameter were taken from the growing edges of 48 h old culture plates using sterilized cork borer.

The essential oils of the root bark and leaves were isolated by steam distillation as discussed under chapters III and IV respectively.

Benzoic acid used in this investigation was obtained from U. narum by extraction with ethanol and fractionating

the extract as explained under section 2 of chapter II.

Benzyl benzoate used in growth study was an annalar commercial sample (Qualigens).

For preparing carrot agar medium, carrot was lightly cooked until tender in sterile distilled water, ground into a paste and diluted with more water, filtered and added agar to it.

Growth study

In the growth study, the extracts (leaf and root bark), benzene and ethyl acetate fractions of alcohol extract, essential oils, benzoic acid or benzyl benzoate were incorporated with carrot agar medium in different concentrations. The Phytophthora capsici was allowed to grow in the above test and control media (carrot agar alone). The diameter of the colony in both were measured and the per cent inhibition to growth was deduced using the formula,[268]

$$\text{Per cent inhibition} = a-b/a \times 100,$$

where 'a' is the radial growth of the colony in the control medium and 'b', the radial growth in the test medium.

Sporulation study

P. capsici culture discs of 0.5 cm were placed in 15 mL solution containing different concentrations of benzoic acid. In control, culture discs were placed in sterile double distilled water. Incubated under luminescent light for sporulation at 20-22°C for 48h. Number of sporangia produced in test media per microscopic field were counted, compared with control and inhibition per cent was calculated using the formula,

$$\text{Per cent inhibition} = a-b/a \times 100,$$

where 'a' is the number of sporangia in the control medium and 'b', the number of sporangia in the test medium.

Zoospore liberation study

To study the effect of benzoic acid on zoospore release, the sporulating discs were taken in Petriplates and solutions of different concentrations of benzoic acid were placed over them. The treated discs were given a cold shock at 10°C and later incubated at room temperature at 28°C. Observations such as number of empty sporangia, sporangia with unliberated zoospores were taken. In control, culture discs were treated with distilled water and given a cold shock. Comparing with control the inhibition per cent was

calculated using the formula,

Per cent inhibition = a-b/a x 100,

where 'a' is the number of empty sporangia in the control and 'b' the number of empty sporangia in the test medium.

Zoospore germination study

In order to study the effect of benzoic acid on zoospore germination of P. capsici, benzoic acid solutions of different concentrations were added to zoospore suspension of P. capsici. Incubated for one day and zoospore germination per microscopic field from each Petriplate was recorded. Zoospore suspension in distilled water served as control. Compared with control and inhibition per cent was calculated using the formula,

Per cent inhibition = a-b/a x 100,

where 'a' is the number of zoospores germinated in the control and 'b' the number of zoospores germinated in the test medium.

Fungicidal and fungistatic activity

Carrot agar medium containing different concentrations

of the test compounds were poured into Petriplates and inoculated with 0.5 cm culture discs of P. capsici and incubated at room temperature. After 72 h, the culture discs were transferred into fresh carrot agar medium and incubated further for 72 h. Growth in the medium was indicative of fungistatic activity while no growth indicated fungicidal activity.

RESULTS AND DISCUSSION

P. capsici has four different phases of growth namely vegetative growth, sporangial formation, zoospore liberation and zoospore germination. If any compound inhibits any one of these four phases, it could be useful as an antifungal agent against this fungus.

Effect of aqueous extract of U. narum leaves and root bark on the growth of P. capsici

The aqueous leaf extract of U. narum was found inhibitory to the fungus at all concentrations tested. At 5 mL/100 mL of medium the per cent inhibition was 70.1. The per cent inhibition at 10,15 and 20 mL/100 mL of medium was 77.85, 90.1 and 100 respectively. Root bark extract of U. narum also showed moderate inhibition against P. capsici but inhibition per cent was less compared to leaf extract. At

5, 10, 15 and 20 mL of the root bark extract per 100 mL of carrot agar medium, the per cent inhibition was 28.5, 36.6, 65.3 and 67.3 respectively (Table V.I).

Table V.I. Inhibitory effect of U. narum leaf and root bark extracts on the growth of P. capsici

Aqueous extract dose (mL/100 mL)	Leaf extract		Root bark extract	
	Radial growth (mm)	Inhibition (%) (a-b/a) x 100	Radial growth (mm)	Inhibition (%) (a-b/a) x 100
Control	32.60	--	32.60	--
5	9.75	70.1	23.3	28.5
10	7.22	77.85	20.67	36.6
15	3.22	90.12	11.3	65.3
20	0	100	10.67	67.3

Effect of benzene fraction of alcohol extract of U. narum leaves on the growth of P. capsici

Benzene fraction of alcohol extract showed 100 per cent inhibition to the growth of P. capsici at a concentration of 2 mg/mL of the extract. The inhibition per cent at 0.4, 0.8, 1.2 and 2 mg/mL of the extract was found to be 26.7, 53.1, 74.35 and 100 respectively (Table V.2).

Effect of ethyl acetate fraction of alcohol extract of U. narum leaves on the growth of P. capsici

Ethyl acetate fraction of alcohol extract of U. narum leaves was found inhibitory to the growth of P. capsici, but the inhibition per cent was less compared to the benzene extract at the same concentration. At a concentration of 2 mg/mL of the medium, the inhibition per cent was about 62 only. Per cent inhibition at concentration 0.4, 0.8, 1.2 and 2 mg/mL of the medium was found to be 9.6, 19, 51.5 and 62.2 respectively (Table V.2).

Table V.2. Effect of benzene and ethyl acetate fraction of alcohol extract of U. narum leaves on the growth of P. capsici

Extract dose (mg/mL)	Benzene extract		Ethyl acetate extract	
	Radial growth (mm)	Inhibition (%) (a-b/a) x 100	Radial growth (mm)	Inhibition (%) (a-b/a) x 100
Control	42.62	--	42.62	--
0.4	31.23	26.7	38.52	9.6
0.8	20.0	53.1	34.51	19.0
1.2	10.93	74.35	20.65	51.5
2	0	100	16.11	62.2

Effect of leaf and root bark essential oil of U. narum on the growth of P. capsici

The leaf essential oil was found to be highly inhibitory to the fungus at a concentration of 1 mg/mL of carrot agar medium. At concentrations of 0.2, 0.4, 0.6 and 0.8 mg/mL of the medium, the inhibition per cent was 24.6, 45.2, 72.7, 90.5, 100 respectively (Table V.3). Root bark essential oil also showed inhibition, but to a lesser extent compared to leaf essential oil at the same concentrations. At concentrations 0.2, 0.4, 0.6, 0.8 and 1 mg/mL of the medium the inhibition per cent of root bark essential oil on P. capsici was 12, 29.6, 47.3, 58.3 and 71.3 respectively (Table V.3).

Table V.3. Effect of leaf and root bark essential oil of U. narum on the growth of P. capsici

Essential oil dose (mg/mL)	Leaf essential oil		Root bark essential oil	
	Radial growth (mm)	Inhibition (%) (a-b/a) x 100	Radial growth (mm)	Inhibition (%) (a-b/a) x 100
Control	52.73	--	52.73	--
0.2	39.75	24.6	46.4	12
0.4	28.9	45.2	37.11	29.6
0.6	14.4	72.7	27.8	47.3
0.8	5	90.5	22	58.3
1.0	0	100	15.13	71.3

Effect of benzoic acid isolated from U. narum leaves on the growth of P. capsici

Benzoic acid was found strongly inhibitory to the growth of P. capsici. At a concentration of 0.5 μ mol/mL (61 ppm), the inhibition per cent was 55 and it increased with increase in concentration of benzoic acid. At concentrations 0.6, 0.7, 0.8, 0.9 and 1 μ mol/mL, the inhibition per cent was 60.4, 86.6, 93.3, 94.9 and 100 respectively (Table V.4).

Table V.4. Effect of benzoic acid isolated from U. narum leaves on the growth of P. capsici

Benzoic acid dose (μ mol/mL)	Radial growth (mm)	Inhibition (%) (a-b/a) x 100
Control	37.25	--
0.5	16.83	54.8
0.6	14.75	60.4
0.7	5.00	86.57
0.8	2.5	93.3
0.9	1.92	94.85
1	0.00	100.00

Effect of benzyl benzoate on the growth of P. capsici

Benzyl benzoate also inhibited the growth of P. capsici, but to a lesser extent compared to benzoic acid. The inhibition per cent was 58.5 at 1 μ mol/mL, 77 at 2 μ mol/mL and 95 at 3 μ mol/mL. Complete inhibition was noticed at 4 μ mol/mL (Table V.5).

Table V.5. Effect of benzyl benzoate on the growth of P. capsici

Benzyl benzoate dose (μ mol/mL)	Radial growth (mm)	Inhibition (%) (a-b/a) x 100
Control	37.25	--
1	15.5	58.4
2	8.5	77.18
3	1.7	95.44
4	0	100

Effect of benzoic acid on sporulation of P. capsici

Sporulation of P. capsici was inhibited even at a very low concentration of benzoic acid. At 0.1 μ mol/mL the inhibition per cent was 28.86. But at 0.2 μ mol/mL the

inhibition per cent increased to 100. At all higher concentrations tested (0.3-0.4 μ mol/mL), there was complete inhibition to sporulation (Table V.6).

Table V.6. Effect of benzoic acid on sporulation of *P. capsici*

Benzoic acid dose (μ mol/mL)	Sporangia/microscopic field (number)	Inhibition (%) (a-b/a) x 100
Control	356	--
0.1	253.25	28.86
0.2	0	100
0.3	0	100
0.4	0	100

Effect of benzoic acid on zoospore liberation of *P. capsici*

Benzoic acid inhibited zoospore liberation of *P. capsici* at low concentrations. Inhibition per cent was found to be 97.86 and 100 at 0.9 μ mol/mL and 1 μ mol/mL respectively. At concentrations 0.1-0.8 μ mol/mL the inhibition per cent was 85.2, 95.45, 95.6, 97.14, 86.8, 87.2, 94.6, 97.3, respectively (Table V.7).

Table V.7. Effect of benzoic acid on zoospore liberation of P. capsici

Benzoic acid dose (μ mol/mL)	Sporangia liberated (number)	Inhibition (%) (a-b/a) x 100
Control	196.0	--
0.1	29.0	85.2
0.2	8.9	95.45
0.3	8.6	95.6
0.4	5.6	97.14
0.5	25.9	86.78
0.6	25.0	87.2
0.7	10.6	94.6
0.8	5.3	97.3
0.9	4.2	97.86
1	0	100

Effect of benzoic acid on zoospore germination of P. capsici

Benozoic acid inhibited zoospore germination of P. capsici even at very low concentration. The inhibition per cent at 0.1, 0.2, 0.3, 0.4, μ mol/mL were 12.4, 18.8, 26.9, and 47.2. But at a concentration of 0.5 μ mol/mL, the

inhibition per cent increased to 99.65 and complete inhibition was noticed at 0.6 μ mol/mL (Table V.7).

Table V.7. Effect of benzoic acid on zoospore germination of P. capsici

Benzoic acid dose (μ mol/mL)	Zoospore germinated (number)	Inhibition (%) (a-b/a) x 100
Control	78.8	--
0.1	69	12.44
0.2	64.0	18.8
0.3	58.0	26.4
0.4	41.6	47.2
0.5	0.5	99.65
0.6	0	100
0.7	0	100
0.8	0	100
0.9	0	100
1	0	100

Fungicidal or fungistatic activity

Benzoic acid was found fungistatic to P. capsici up to 2 μ mol/mL and fungicidal at higher concentrations.

However, benzyl benzoate was fungistatic at all concentrations tested.

The present investigation established that the aqueous extract of the leaves of U. narum is highly inhibitory to the soilborn fungus P. capsici. Benzoic acid, isolated from U. narum leaves inhibited all the four phases of growth of P. capsici to hundred per cent at concentrations ranging from 0.2 μ mol/mL to 1 μ mol/mL, establishing its effectivenss as a fungicide. Benzyl benzoate, an important constituent of the leaf essential oil was also found to be antagonistic to this fungus, but to a lesser extent. Since U. narum is used as a green manure, its use as a mulch which decay slowly releasing the antifungals into the soil may help in checking P. capsici in soil. Also, since benzoic acid is a cheap chemical, it may be used against the foot rot disease which requires more investigation.

Even though benzoic acid is a well known antifungal agent, its isolation from U. narum is reported for the first time. Detailed investiation on the effect of benzoic acid on various phases of P. capsici are new and significant. The steep fall in antifungal activity when benzoic acid is replaced by benzyl benzoate signifies the importance of

-COOH group for this property. Benzoic acid was found to be fungistatic up to a concentration of 2 μ mol/mL and fungicidal above this concentration, where as benzyl benzoate was only fungistatic. This observation prompted the investigation on structure-activity relationship given in the next section.

EXPERIMENTAL

Water used in all the experiments involving fungi was double distilled using a glass apparatus.

Preparation of aqueous extract of U. narum leaves

Leaves of U. narum were collected from the Calicut University Campus. Fresh plant material was washed well in water. Dried in an oven at 45°C and finely powdered using a mixer grinder. The powdered material (100 g) was then refluxed with 600 mL sterile distilled water for 3 h, kept as such overnight and filtered. Total volume of the extract was then reduced to 100 mL by evaporation on a sand bath. This solution was considered as 100% extract.

Preparation of aqueous extract of U. narum root bark

U. narum roots were collected from Calicut University

Campus and washed well in water. Barks were separated using a knife and dried in an oven at 45°C and finely powdered using a mixer grinder. 100 g of the powdered material was then refluxed with 600 mL sterile distilled water for 3 h. Kept as such overnight and filtered. Total volume of the extract was then reduced to 100 mL by evaporation on a sand bath. This solution was considered as 100% extract.

Preparation of P. capsici cultures

The pure culture of P. capsici was placed at the centre of Petriplates containing carrot agar medium and incubated in dark at 20-22°C. Culture discs for experiment were cut out from the growing edge after 48 h.

Preparation of U. narum leaf essential oil

Leaves of U. narum were collected from the Calicut University Campus. The shade dried leaves (800 g) were powdered in an electric mixer grinder and steam distilled for 5 h. The distillate so obtained was extracted with solvent ether (3 x 100 mL). Ether portions were pooled together and dried with anhydrous sodium sulphate. On evaporation of dry ether extract, after removal of sodium sulphate, over a water bath yielded 0.9 g (0.11%) of the oil.

Preparation of U. narum root bark essential oil

U. narum roots were collected from the Calicut University Campus. Barks were separated with a knife and washed well, dried in an oven at 45°C, powdered using a mixer grinder (250 g) and steam distilled for 5 h. The distillate was extracted with solvent ether (3 x 100 mL). Combined the ether portion and dried with anhydrous sodium sulphate. Evaporation of dry ether extract after removal of sodium sulphate, over a water bath yielded 0.65 g (0.26%) of oil.

Isolation and Identification of benzoic acid from U. narum leaves

Dried and finely powdered leaves (2 kg) of U. narum were extracted repeatedly with petroleum spirit (60-80°C, 3 x 6 L) and then with ethyl alcohol (6 L). The alcohol extract was then concentrated to about 250 mL under reduced pressure and added about 250 mL of distilled water. This aqueous solution was then fractionated with benzene and ethyl acetate. Screening of the petroleum ether, benzene and ethyl acetate extracts for their antifungal activity showed that benzene extract was highly inhibitory to P. capsici. Column chromatography of the benzene extract lead

to the isolation of a colourless crystalline compound, melting at 119^{o}C. This compound was identified as benzoic acid from its various spectral data (Chapter II, Section:2).

Preparation of media for antifungal studies

Carrot (300 g) was cooked lightly until tender in 500 mL distilled water. The cooked carrot was ground into a paste using a mixer grinder and added 1000 mL more water. After filtration through a muslin cloth, 30 g of agar (BDH) was added to the carrot preparation and shaken to get a homogeneous mixture.

Growth study

In a preliminary study, the extracts were added to carrot agar medium before and after autoclaving and the growth of P. capsici was tested. There was inhibition of growth in both the plates inoculated with the fungus where the extracts were added before and after autoclaving indicating the thermostable nature of the extracts. Hence all further studies were conducted by adding extracts and then autoclaving the medium.

Antifungal activity of aqueous extract of U. narum leaves

Different volumes of aqueous extract of U. narum leaves (5,10,15,20 mL) were mixed with carrot agar medium (95, 90, 85 and 80 mL) to obtain desired concentrations (5%, 10%, 15%, 20%) in the final volume of 100 mL of medium. In control sets appropriate quantities of sterile double distilled water (5, 10, 15 and 20 mL) were mixed in place of test solution to obtain 100 mL carrot agar medium. These solutions were autoclaved at 15 psi for 30 minutes. After cooling to 40^{o}C, each medium was dispensed into four Petriplates (90 mm) for replication. Mycelial discs of 0.5 cm taken from the growing edges of 48h old culture of P. capsici were placed in the centre of these Petriplates. The plates were incubated in dark at 20-22^{o}C for 72 h. The radial growth of mycelium in each Petriplate was measured at two points along the diameter of the plate and the mean of these two readings taken as the radial growth of the colony. The growth of the colony in the control sets was compared with that of various treatments and converted into per cent inhibition.

Antifungal activity of aqueous extract of U. narum root bark

Different volumes of aqueous extract of U. narum root

bark (5, 10, 15, 20 mL) were mixed with carrot agar medium to obtain disired concentrations (5%, 10%, 15%, 20%) in the final volume of 100 mL of medium. In control sets, appropriate quantities of sterile double distilled water (5, 10, 15 and 20 mL) were mixed in place of test solutions to obtain 100 mL carrot agar medium. These solutions were autoclaved at 15 psi for 30 minutes. After cooling to 40^{o}C each medium was dispensed into four Petriplates (90 mm) for replication. P. capsici culture discs of 0.5 cm, taken from the growing edges of 48 h. old culture plates were placed at the centre of each Petriplate. The plates were incubated in the dark at 20-22^{o}C for 72 h. The radial growth of mycelium in each petriplate was measured at two points along the diameter of the plate and the mean of these two readings taken as the radial growth of the colony. The growth of colony in the conrol sets was compared with that of various treatments and the difference converted into per cent inhibition.

Effect of benzene fraction of alcohol extract of U. narum leaves on the growth of P. capsici

In order to study the effect of benzene fraction of alcohol extract on the growth of P. capsici, the solid matter after removal of benzene (1 g) was dissolved in 50 mL

of acetone. Different volumes (2, 4,6,8, 10 mL) of this solution were then added to carrot agar medium to obtain desired concentrations (0.4, 0.8, 1.2, 1.6, 2 mg/mL) in the final volume of 100 mL of medium. In control sets, above mentioned quantities of acetone were taken in place of test solutions to obtain 100 mL carrot agar medium. The carrot agar media were autoclaved at 15 psi for 30 minutes. After cooling to 40^{o}C, each medium was dispensed into four petriplates (90 mm). Mycelial discs of 0.5 cm taken from the growing edges of 48 h old culture plates of P. capsici were placed at the centre of Petriplates. The plates were incubated in dark at 20-22^{o}C for 72 h. Radial growth of mycelium in each Petriplate was measured at two points along the diameter of the plate and the mean of these two readings was taken as the radial growth of the colony. The growth of the colony in control sets was compared with that of various treatments and the difference converted into per cent inhibition.

Effect of ethyl acetate fraction of alcohol extract of U. narum leaves on the growth of P. capsici

In order to study the effect of ethyl acetate portion of alcohol extract on the growth of P. capsici, ethyl acetate extract was dried well. One gram of this was

dissolved in 50 mL of acetone. Different volumes (2,4, 6, 8, 10 mL) of this solution were then added to carrot agar medium to obtain desired concentrations (0.4, 0.8, 1.2, 1.6 and 2 mg/mL) in the final volume of 100 mL of medium. In control sets, appropriate quanities of acetone were mixed in place of test solution to obtain 100 mL carrot agar medium. After autoclaving at 15 psi for 30 minutes and cooling to 40^{o}C, each medium was dispensed into four Petriplates (90 mm) for replication. Mycelial discs of 0.5 cm diameter taken from the growing edges of 48 h old culture plates of P. capsici were placed in the centre of Petriplates. The plates were incubated in dark at 20-22^{o}C for 72 h. The radial growth in each Petriplate was measured at two points along the diameter of Petriplate and mean of these two readings was taken as the radial growth of the colony. Compared with the growth in the control sets and per cent inhibition was calculated.

Effect of U. narum leaf essential oil on the growth of P. capsici

Leaf essential oil obtained by steam distillation (0.5 g) was dissolved in 50 mL of acetone. Different volumes (1, 2, 3, 4, 5 mL) of this solution were added to carrot agar medium to obtain desired concentrations of (0.2, 0.4, 0.6,

0.8 and 1 mg/mL) of the test compound in the final volume 100 mL of carrot agar medium. For control sets acetone (1, 2, 3, 4, 5 ml) were added to get 100 mL of carrot agar medium. The Petriplates were autoclaved at 15 psi for 30 minutes and cooled to 40^{o}C. The medium was dispensed into four petriplates for replication. Mycelial discs of 0.5 cm taken from the growing edges of 48 h old culture plate of *P. capsici* were placed at the centre of these Petriplates. Incubated in the dark at 20-22^{o}C for 72 h. The radial growth in each Petriplate was measured at two points along the diameter of the plates and the mean of these two readings was taken as the radial growth of the colony. The growth of the colony in the control sets was compared with that of various treatments and difference converted into per cent inhibition.

Effect of *U. narum* root bark essential oil on the growth of *P. capsici*

Root bark essential oil of *U. narum* obtained by steam distillation (0.5 g) was dissolved in 50 mL of acetone. Different volumes (1,2,3,4,5 mL) of this solution were added to get desired concentrations of (0.2, 0.4, 0.6, 0.8 and 1 mg/mL) in the final volume of 100 mL of carrot agar medium. For control sets appropriate quantities of acetone were

added to get 100 mL of carrot agar medium. The media were then autoclaved at 15 psi for 30 minutes. After cooling to 40°C, each medium was dispensed into four Petriplates. Discs of 0.5 cm taken from the growing edges of 48 h old culture plate of P. capsici were placed at the centre of these Petriplates. Incubated in dark at 20-22°C for 72 h. Radial growth in each Petriplate was measured at two points along the diameter of the Petriplates. The mean of these two readings gave the radial growth of the colony. Compared the growth in the control sets with that in various treatments and percent inhibition was calculated.

Effect of benzoic acid on the growth of P. capsici

To study the effect of benzoic acid on the growth of P. capsici, a stock solution of benzoic acid (100 μ mol/mL) was prepared by dissolving 1.2 g of the compound in 100 mL of acetone. Different volumes (0.5, 0.6, 0.7, 0.8, 0.9 and 1 mL) of this solution were added to get desired concentrations (0.5, 0.6, 0.7, 0.8, 0.9 and 1 μ mol/ml) in the final volume of 100 mL carrot agar medium. Control sets were prepared by adding appropriate quantities of acetone (0.5, 0.6, 0.7, 0.8, 0.9 and 1 mL) to get 100 mL of carrot agar medium. After autoclaving at 15 psi for 30 minutes, the culture media were cooled to 40°C and dispensed into four

Petriplates and inoculated with 0.5 cm culture discs taken from the growing edges of 48 h old culture plate of P. capsici in the centre. Incubated in dark at 20-22°C for 72 h. The radial growth of mycelium in each Petriplate was measured at two points along the diameter of the Petriplates and mean of these two readings gave the radial growth of the colony. Compared the growth of the colony in the control sets with that in various treatments and per cent inhibition was calculated.

Effect of benzyl benzoate on the growth of P. capsici

To evaluate the effect of benzyl benzoate on the growth of P. capsici, a stock solution of benzyl benzoate (100 μ mol/mL) was prepared by dissolving 2.12 g (0.01 mol) of the compound in 100 mL of acetone. Different volumes (1,2,3 and 4 mL) of this solution were added to get desired concentrations (1 μ mol/mL-4 μ mol/mL) in the final volume of 100 mL of carrot agar medium. Control sets were prepared by adding appropriate quantities of acetone (1-4 mL) to get 100 mL of carrot agar medium. These media were then autoclaved at 15 psi for 30 minutes. After cooling to 40°C, each medium was dispensed in to four Petriplates (90 mm) for replication. Mycelial discs of 0.5 cm diameter taken from the growing edges of 48 h old culture plates of P. capsici

were placed at the centre of the Petriplates. The plates were incubated in dark at 20-22°C for 72 h. The radial growth of mycelum in each Petriplate was measured at two points along the diameter of the Petriplates and mean of these two readings gave the radial growth of the colony. Compared the growth of the colony in the control sets with that in various treatments and per cent inhibition was calculated.

Sporulation study

To study the effect of benzoic acid on sporulation of P. capsici, a stock solution of benzoic acid (100 μ mol/mL) in acetone was prepared. Different volumes of this solution (0.1-0.4 mL) were diluted with distilled water (100 mL) to obtain desired concentrations (0.1-0.4 μ mol/mL) of test solution. These solutions were boiled to remove acetone. Mycelial discs taken from the growing edges of 48 h old culture plate of P. capsici incubated in dark were placed at the centre of Petriplates, keeping three discs per plate. The above solutions (15 mL) were placed on these discs. In control the discs were covered with sterile double distilled water. All the Petriplates were then incubated under luminescent light for sporulation at 20-22°C for 48 h. Number of sporangia formed per microscopic field were

counted (magnification 15/14 x 4, Nikon). The average of three microscopic fields per disc for three replications were counted and compared with that of control from which per cent inhibition was calculated.

Zoospore liberation study

To study the effect of benzoic acid on zoospore release (indirect germination of sporangia), P. capsici culture grown on carrot agar medium for 48 h in dark at 20-22^{o}C was taken. Mycelial discs of 0.5 cm diameter were cut out from the growing edges of this culture and allowed to sporulate by incubating under luminescent light for 24 h. Such sporulating discs were taken in Petriplates and benzoic acid solutions of concentrations ranging from 0.1 to 0.5 umol/mL placed over them. These treated discs were then given a cold shock at 10^{o}C to facilitate indirect germination and later incubated at room temperature at 28^{o}C for 30 minutes. For control sets distilled water was used instead of benzoic acid solution. Due to cold shock, zoospores formed inside the sporangia are released. The number of empty sporangia were counted from three microscopic fields per disc, three discs per plate and for three replication. Compared with control and per cent inhibition was calculated.

Zoospore germination study

Inorder to evaluate the effect of benzoic acid on zoospore germination, sporulating culture discs of P. capsici were subjected to cold shock at 10°C for 10 minutes as described under zoospore liberation and prepared the zoospore suspension. To this suspension, benzoic acid solution was added to get the desired concentrations (0.1-1 μ mol/mL). After incubating for one day at room temperature, zoospore germination per microscopic field from each Petriplate was recorded. Zoospore suspension in sterile double distilled water served as control. Inhibition of zoospore germination was calculated by comparing with control.

Fungicidal or fungistatic activity

In order to study the fungicidal or fungistatic activity of benzoic acid and benzyl benzoate, carrot agar medium containing different concentrations (1-4 μ mol/mL) of the test compounds were poured into Petriplates after autoclaving and cooling to 40°C. Culture discs of 0.5 cm taken from growing edges of 48 h old cultures of P. capsici were kept in the medium and incubated at room temperature.

After 72 h, the culture discs were transferred into fresh carrot agar medium and incubated further for 72 h. Growth in the medium was indicative of fungistatic activity while no growth indicated fungicidal activity.

SECTION 2: ANTIFUNGAL PROPERTY AND STRUCTURE-ACTIVITY RELATIONSHIP

Studies on structure-activity relationships have given birth to very useful compounds in the fields of medicine and agriculture. From the first section of this chapter, it was observed that benzoic acid is quite inhibitory to P. capsici while benzyl benzoate, a compound structurally related to benzoic acid also exhibited the same property, but to a lesser extent. Hence it was thought worthwhile to investigate the effect of compounds structurally related to benzoic acid on the growth of P. capsici. It had already proved that absence of carboxylic acid group reduced the antifungal activity. Hence seven aromatic carboxylic acids including 2-furoic acid, five aliphatic acids, acetophenone and sodium benzoate were screened for their antifungal activity. Acetophenone was screened so that the effect of benzoyl group can be assessed.

MATERIALS AND METHODS

All the compounds studied except 2-furoic acid, for their activity were commercially available (Fluka) annalar samples.

2-Furoic acid was prepared from 2-furaldehyde. For this 2-furaldehyde (1.9 g) was dissolved in pyridine (5 mL) and cooled in ice. A solution of potassium permanganate (2.1 g) in pyridine (28 mL) and water (12 mL) was then added with rapid stirring. The mixture was stirred for 30 minutes, filtered and boiled off the solvent under suction. The dried residue was then acidified with dilute hydrochloric acid and extracted with ether. Ether extract was dried with anhydrous sodium sulphate. Evaporation of dry ether extract over a water bath yielded crystals of furoic acid m.p. 136^{o}C.[268]

To evaluate the efficacy of the individual compounds on the growth of P. capsici, carrot agar medium with concentrations of test compounds ranging from 0.1 μ mol/mL to 4 μ mol/mL were prepared. After inoculation of P. capsici in these media, its growth was meaured after incubation at 20-22^{o}C in dark for 72 h. The per cent inhibition to growth was calculated as in the previous section using the equation,

$$\text{Per cent inhibition} = a-b/a \times 100,$$

Where, 'a' is the radial growth of the colony in the control medium and 'b' the radial growth in the test solution.

In cases where the per cent inhibition was very low for 1 μ mol/mL concentration, higher concentrations were investigated. In other cases lower concentrations were investigated for their activity. In the case of acetic acid, acetophenone and propionic acid a considerable decrease in activity was noticed when the solution were incorporated before autoclaving when compared to that added after autoclaving. Hence these compoundswere incorporated into medium only after autoclaving.

RESULTS AND DISCUSSION

The antifungal activities of the compounds investigated, phenylacetic acid, naphthoic acid, 4-methyl benzoic acid, 2-methylbenzoic acid, 3-methylbenzoic acid, 2-hydroxybenzoic acid, furoic acid, acetophenone, acetic acid, propionic acid, oxalic acid, malonic acid, succinic acid and sodium benzoate are summarized in the table V.2.1. For the sake of comparison, the antifungal activity of benzoic acid also is given in the same table. It was found that substitution with methyl groups at ortho, meta or para positions of benzene ring did not alter the activity remarkably. However, salicylic acid with hydroxyl group at ortho position is remarkably less inhibitory. This can be

attributed to the intramolecular hydrogen bonding in salycilic acid which alters the bonding nature in carboxylic acid group. Furoic acid was found to be inferior in activity compared to other aromatic carboxylic acids. Naphthoic acid and phenylacetic acid also were comparable to benzoic acid in their activity. This shows the requirement of both an aromatic ring and carboxylic acid group for the antifungal property. Acetophenone was found to have very low antifungal activity proving that the benzoyl group alone cannot serve this purpose.

All the aliphatic acids screened were quite inferior to aromatic acids, justifying the combination of aromatic ring and carboxylic acid group for the activity. Sodium benzoate, a commonly used food perservative was also found to be less effective. This observation underscores the significance of the carboxylic acid group in eliciting antifungal activity against P. capsici.

Table V.2.1. Activity of aromatic and aliphatic acids, acetophenone and sodium benzoate against P. capsici

Sample	Dose (μ mol/mL)	Radial growth (mm)	Inhibition (%) (a-b/ a x 100
Benzoic acid	Control	37.25	--
	0.5	16.83	54.8
	0.6	14.75	60.4
	0.7	5	86.57
	0.8	2.5	93.29
	0.9	1.92	94.85
	1	0	100
Phenylacetic acid	Control	37.25	--
	0.5	17.8	52.2
	0.6	5.75	84.56
	0.7	5.33	85.69
	0.8	5.08	86.36
	0.9	2.33	93.75
	1	0	100
Naphthoic acid	Control	45.94	--
	0.5	20.23	55.96
	0.6	14.72	67.96
	0.7	5.85	87.27
	0.8	3.37	92.66
	0.9	0.89	98.06
	1	0	100
4-Methylbenzoic acid	Control	45.94	--
	0.5	26.71	41.86
	0.6	12.22	73.4
	0.7	6.31	86.26
	0.8	3.15	93.14
	0.9	1.78	96.13
	1	0	100

Sample	Dose (μ mol/mL)	Radial growth (mm)	Inhibition (%) (a-b/ a x 100
2-Methyl-benzoic acid	Control	45.94	--
	0.5	29.2	36.43
	0.6	17.64	61.6
	0.7	9.33	79.7
	0.8	4.41	90.4
	0.9	1.76	96.17
	1	0	100
3-Methyl-benzoic acid	Control	45.94	--
	0.5	27.12	40.97
	0.6	18.25	60.28
	0.7	11.2	75.62
	0.8	4.3	90.64
	0.9	2.26	95.08
	1	0	100
2-Hydroxy-benzoic acid	Control	45.94	--
	0.5	37.36	18.68
	0.6	22.14	51.8
	0.7	19.62	57.29
	0.8	14.33	68.8
	0.9	11.22	75.58
	1	8.44	81.62
2-Furoic acid	Control	61.25	--
	1	27.22	55.56
	2	10.97	82.09
	3	6.26	89.77
	4	3.71	93.94
Acetophenone	Control	61.25	--
	1	32.33	47.2
	2	21.46	64.96
	3	16.75	72.65
	4	9.61	84.3

Sample	Dose (μ mol/mL)	Radial growth (mm)	Inhibition (%) (a-b/ a x 100
Acetic acid	Control	66.71	--
	1	57.12	14.38
	2	42.37	36.49
	3	36.14	45.83
	4	19.29	71.08
Propionic acid	Control	66.71	--
	1	59.40	10.96
	2	47.64	28.59
	3	39.44	40.88
	4	22.65	66.05
Oxalic acid	Control	75	--
	1	62.41	16.79
	2	43.35	42.2
	3	36.71	51.05
	4	22.85	69.53
Malonic acid	Control	75	--
	1	66.75	11.07
	2	51.23	31.69
	3	37.69	49.75
	4	28.43	62.09
Succinic acid	Control	75	--
	1	70.5	6
	2	63	16
	3	57.1	23.9
	4	39.65	47
Sodium benzoate	Control	75	--
	1	54.14	27.81
	2	29.77	60.31
	3	10.33	86.2
	4	6.14	92.96

EXPERIMENTAL

1. Effect of Phenylacetic acid on the growth of P. capsici

Phenylacetic acid solution of 0.01 molarity (100 μ mol/mL) was prepared by dissolving 1.36 g of phenylacetic acid in acetone (100 mL). Different volumes of this solution (0.5, 0.6, 0.7, 0.8, 0.9 and 1 mL) were pipetted out into conical flasks containing 100 mL carrot agar and shaken to homogenize so as to get carrot agar media containing 0.5, 0.6, 0.7, 0.8, 0.9 and 1 μ mol of phenylacetic acid per one mL of media. After autoclaving, each of the above culture media were poured into four Petriplates for replication and inoculated with P. capsici culture discs of 0.5 cm diameter taken from the growing edges of 48 h old culture plate of P. capsici grown in dark at 20-22°C. All the Petriplates were then incubated in dark at 20-22°C for 72 h. Growth of the colony in each Petriplate was measured at two points along the diameter of the plate. Mean of these two readings was taken as the radial growth of the colony. The growth of colony in the test media was compared with that in control sets and per cent inhibition was calculated.

Culture media for all the other compounds tested were

prepared exactly as in the case of phenylacetic acid except for the concentrations.

2. Effect of naphthoic acid on the growth of P. capsici

Naphthoic acid solution of 0.01 molarity (100 μ mol/mL) was prepared by dissolving 1.72 g of it in acetone (100 mL). Different volumes of this solution (0.5, 0.6, 0.7, 0.8, 0.9 and 1 mL) were incorporated with carrot agar medium before autoclaving and the experiment was conducted as in the first case.

3. Effect of 4-methylbenzoic acid on the growth of P. capsici

4-Methylbenzoic acid solution of 0.01 molarity (100 μ mol/mL) was prepared by dissolving 1.36 g of it in acetone (100 mL). Different volumes of this solution (0.5, 0.6, 0.7, 0.8, 0.9 and 1 mL) were incorporated with carrot agar medium before autoclaving and the experiment was conducted as in the first case.

4. Effect of 2-methylbenzoic acid on the growth of P. capsici

2-Methylbenzoic acid solution of 0.01 molarity (100 μ

mol/mL) was prepared by dissolving 1.36 g of it in acetone (100 mL). Different volumes of this solution (0.5, 0.6, 0.7, 0.8, 0.9 and 1 mL) were incorporated with carrot agar medium before autoclaving and experiment was conducted as in the first case.

5. **Effect of 3-methylbenzoic acid on the growth of P. capsici**

3-Methylbenzoic acid solution of 0.01 molarity (100 μ mol/mL) was prepared by dissolving 1.36 g of it in acetone (100 mL). Different volumes of this solution (0.5, 0.6, 0.7, 0.8, 0.9 and 1 mL) were incorporated with carrot agar medium before autoclaving and the experiment was conducted as in the first case.

6. **Effect of 2-hydroxybenzoic acid on the growth of P. capsici**

2-Hydroxybenzoic acid solution of 0.01 molarity (100 μ mol/mL) was prepared by dissolivng 1.38 g of it in acetone (100 mL). Different volumes of this solution (0.5, 0.6, 0.7, 0.8, 0.9 and 1 mL) were incorporated with carrot agar medium before autoclaving and the experiment was conducted as in the first case.

7. Effect of 2-furoic acid on the growth of P. capsici

2-Furoic acid solution of 0.01 molarity (100 μ mol/mL) was prepared by dissolving 1.12 g of it in acetone (100 mL). Different volumes (1, 2, 3 and 4 mL) of this solution were incorporated with carrot agar medium before autoclaving and the experiment was conducted as in the first case.

8. Effect of acetophenone on the growth of P. capsici

Acetophenone solution of 0.01 molarity (100 μ mol/mL) was prepared by dissolving 1.2 g of it in acetone (100 mL). Different volumes (1, 2, 3 and 4 mL) of this solution were incorporated with 99, 98, 97 and 96 mL of carrot agar media after autoclaving. In control sets 1, 2, 3 and 4 mL of acetone were added to 99, 98, 97 and 96 mL of medium after autoclaving and the experiment was conducted as in the first case.

9. Effect of acetic acid on the growth of P. capsici

Acetic acid solution of 0.01 molarity (100 μ mol/mL) was prepared by dissolving 0.6 g of it in sterile water (100 mL). Different volumes of this solution (1, 2, 3 and 4 mL) were incorporated with 99, 98, 97 and 96 mL of carrot agar medium after autoclaving. In control sets also 1, 2, 3 and

4 mL of sterile water was added to 99, 98, 97 and 96 mL of carrot agar medium after autoclaving and the experiment was conducted as in the previous case.

10. Effect of propionic acid on the growth of P. capsici

Propionic acid solution of 0.01 molarity (100 μ mol/mL) was prepared by dissolving 7.4g of it in acetone (100 mL). Different volumes (1, 2, 3 and 4 mL) of this solution were incorporated with 99, 98, 97 and 96 mL of carrot agar medium after autoclaving. In control sets 1, 2, 3 and 4 mL of acetone was incorporated with 99, 98, 97 and 96 mL of carrot agar medium after autoclaving and the experiment was conducted as in the first case.

11. Effect of oxalic acid on the growth of P. capsici

Oxalic acid solution of 0.01 molarity (100 μ mol/mL) was prepared by dissolving 0.9 g of it in acetone (100 mL). Different volumes of this solution (1, 2, 3 and 4 mL) were incorporated with carrot agar medium (100 mL) and experiment was conducted as in the first case.

12. Effect of malonic acid on the growth of P. capsici

Malonic acid solution of 0.01 molarity (100 μ mol/mL)

was prepared by dissolving 1.04 g of it in 100 mL acetone. Different volumes of this solution (1, 2, 3 and 4 mL) were incorporated with carrot agar medium (100 mL) and the experiment was conducted as in the first case.

13. Effect of Succinic acid on the growth of P. capsici

Succinic acid solution of 0.01 molarity (100 μ mol/mL) was prepared by dissolving 1.18 g of it in acetone (100 mL). Different volumes of this solution (1, 2, 3 and 4 mL) were incorporated with carrot agar medium (100 mL) before autoclaving and experiment was conducted as in the first case.

14. Effect of sodium benzoate on the growth of P. capsici

Sodium benzoate solution of 0.01 molarity (100 μ mol/mL) was prepared by dissolving 1.44 g of it in 100 mL acetone. Different volumes of this solution (1, 2, 3 and 4 mL) were incorporated with carrot agar medium (100 mL) before autoclaving and the experiment was conducted as in the first case.

S U M M A R Y

The work presented in this Thesis is summarised below:

The chemical investigation on Hibiscus furcatus leaves resulted in the isolation and identification of triacontane, hentriacontane, dotriacontane, tritriacontane, tetratriacontane, friedelin, taraxerol, 3-sitosterol, octacosanol, nonacosanol, triacontanol, hentriacontanol, dotriacontanol, tritriacontanol and tetratriacontanol. The results have been published in Asian J. Chem., (1997), 9(4), 134, as an article entitled 'Chemical Investigation on the leaves of Hibiscus furcatus'.

Studies on Uvaria narum lead to the isolation and identification of nonacosane, hentriacontane, tritriacontane, octacosanol, triacontanol, dotriacontanol, tetratriacontanol, 3-sitosterol and benzoic acid. Another compound, dihydrokaempferol 4'-methyl ether also was tentatively characterized.

The analysis of Uvaria narum leaf essential oil revealed the presence of sixty six compounds in it. The odour characteristics of this oil could be explained on the basis of the constituents present. Based on this work an article entitled "Volatiles of the Essential Oils of the

Leaves of Uvaria narum Wall (Annonaceae)' has been presented in J. Essent. Oil. Res. (1997), 9, 217.

The reinvestigation of root bark essential oil of Uvaria narum culminated in the identification of fifty seven compounds of which forty were identified for the first time. The odour characteristics of this oil could be explained on the basis of the constituents present. The medicinal property of this essential oil could also be attributed to the components present. Based on this work an article entitled 'Analysis of the Volatile Constituents of the Root Bark Essential Oil of Uvaria narum Wall by GC-MS and GC-FTIR-MS' has been published in the journal, Sci. Pharm. (1996), 64, 287.

The leaf extract of Uvaria narum was found to be highly inhibitory to the fungus Phytophthora capsici. The most active principle from the leaves was isolated and identified to be benzoic acid. Benzoic acid inhibited all the four phases of growth of P. capsici. This work has been published in Allelopathy Journal, (1998), 5(1), 67, the title of the article being 'Antifungal activity of Uvaria narum extracts'. Studies on structure-activity relationships proved that high activity against P. capsici is exhibited by aromatic carboxylic acids.

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